ON THE MECHANISM OF OPSONIN AND BACTERIOTROPIN ACTION

V. Experimental Test of a Theory of Tropin Action

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In previous papers we have reported that sera which have promoted the phagocytosis of acid-fast bacteria have regularly produced certain alterations in the bacterial surfaces (1, 2, 3) in corresponding degree. Conversely sera which produced these changes in the bacterial surfaces promoted phagocytosis correspondingly, although certain exceptions to the the latter rule were found in using sera which had been altered by heating or aging. Both surface and tropin effects were clearly due to the deposit of certain substances from the immune sera on the surfaces of the homologous bacterial antigen; heating or aging in some cases impaired the phagocytosis-promoting action of these substances without similarly impairing their capacity to combine with the bacteria. The correspondence between the effectiveness of fresh rabbit immune sera in producing surface changes and in promoting phagocytosis was found to hold with sera of various strengths, during the course of immunization, and during the course of tuberculous infection and reinfection.

It then became of interest to determine the distribution of the substances responsible for the surface and tropin effects in the several fractions of immune sera. This subject is treated in the present paper.

Methods

Fractionation of Sera

Fresh serum, without preservative, is diluted with an equal volume of 0.85 per cent NaCl solution. To one volume of serum, diluted, one volume of 66 per cent

saturated ammonium sulfate solution is added. The mixture is shaken thoroughly and allowed to stand at room temperature for 15 minutes. It is then filtered in the ice box using a rather thick, soft filter paper. To the clear filtrate enough ammonium sulfate is added to bring the concentration of ammonium sulfate to 50 per cent. This mixture is shaken thoroughly and allowed to stand 15 minutes at room temperature and filtered in the ice box. To the clear filtrate again enough ammonium sulfate is added to obtain a saturated solution; this is shaken thoroughly and after standing 15 minutes at room temperature, filtered in the ice box. The first precipitation separates the "euglobulin" fraction; the second the "pseudoglobulin" fraction, and in the third the albumin is brought down. After the filtration of each fraction the dried filter paper is temporarily stored in large petri dishes in the ice box. The precipitates are dissolved and washed off from the filter paper, using liberal amounts of distilled water. The solutions are then dialyzed for 4 days in the ice box, using parchment thimbles, and large quantities of water which are frequently changed. When dialysis is completed the fractions are evaporated to the original volume, with the aid of an electric fan, and enough sodium chloride is added to make 0.85 per cent solutions. If the solutions are not completely clear, centrifugation may be resorted to. If it is desired to separate the hydrophobic pseudoglobulins from the hydrophilic portion, after dialysis of the pseudoglobulin fraction, the contents of the dialyzing bag are centrifugalized at once. The sediment of hydrophobic pseudoglobulin can thus be separated by decantation.

Testing of Surface and Phagocytosis-Promoting Effects

The methods used in testing the effects of the sera and serum fractions were those already described in an earlier paper (1).

An even suspension of each antigen* was made and adjusted to a standard turbidity. Serial dilutions of each serum and serum fraction to be used were made. 1 cc. of antigen suspension was mixed with each dilution of serum or serum fraction. All tubes stood at room temperature usually for 30 to 90 minutes and were then put in the ice box over night. In the morning *agglutination* or sedimentation was read without shaking up the tubes. All tubes were centrifuged until clear; the supernatants were decanted, two drops of 0.85 per cent sodium chloride solution were added to each tube and all tubes were shaken up in the *resuspension* test. Excess of saline solution was added to each tube and all were again centrifuged. The supernatant fluids were again decanted, and the sensitized sediments brought to as even a suspension as possible in their original volume of saline. These suspensions of washed sensitized antigen were routinely used for *cataphoresis* and *interface* reactions, and for the tests of phagocytosis of washed antigen.

* The term "antigen" is for convenience applied in this paper to the whole bacteria; it is of course understood that the bacterial cell usually contains more than one antigen.

Phagocytosis of washed antigen was tested by mixing 0.2 cc. of polymorphonuclear leucocyte suspension with 0.2 cc. of each washed, sensitized antigen suspension in a vial and rotating all the vials for 15 minutes on a Robertson agitator. Phagocytosis in the presence of serum or serum-fraction dilution was conducted by rotating in each vial a mixture of 0.2 cc. of leucocytes suspension, 0.1 cc. of serum dilution and 0.1 cc. of stock antigen suspension (of twice the concentration of the washed suspension mentioned above). The making of smears, staining and counting in the phagocytosis tests were as already described.

Certain minor departures from the routine outlined were made from time to time, but these were unimportant. For instance resuspension and cataphoresis were occasionally tested with the particles suspended both in the original serum or serum fraction dilutions and also after washing.

Increased cohesiveness of the antigen surface due to sensitization is indicated by positive resuspension and interface reactions. Decreased surface electric potential difference is indicated by decreased velocity of migration in cataphoresis. Altered wetting properties of the antigen surface are indicated by the interface reaction. The degree of phagocytosis-promoting or tropin effect is indicated by comparison of the percentage phagocytosis of sensitized and control bacterial suspensions.

Correlation between Bacterial Surface and Tropin Effects Produced by Immune Sera and Their Fractions

The immune sera and their fractions have proved to be effective in the same order in altering the surface and in causing agglutination and phagocytosis of their homologous bacteria. The order of effectiveness has been uniformly: whole serum > euglobulin > pseudoglobulin > albumin. In only one of eleven experiments was any deviation from this order detected; in this experiment euglobulin was more effective than pseudoglobulin in some reactions, less effective in others.

As heretofore the correspondence between the surface changes and the agglutination and phagocytosis brought about by whole immune sera has been found in this work to be exceedingly close. The correspondence between surface, agglutinating and bacteriotropic effects has been exceedingly good also when the euglobulin or pseudoglobulin fractions of immune sera have been used. Of course the parallelism between surface and tropin effects was not perfect in all cases; for

instance the phagocytosis titers tended to run a little higher than those of the surface reactions when Arloing, Prague or chelonei strains were used as antigens, a little lower when Bovine III was antigen; also minor quantitative discrepancies occurred from time to time. Such occasional slight discrepancies are, however, inevitable in an extensive

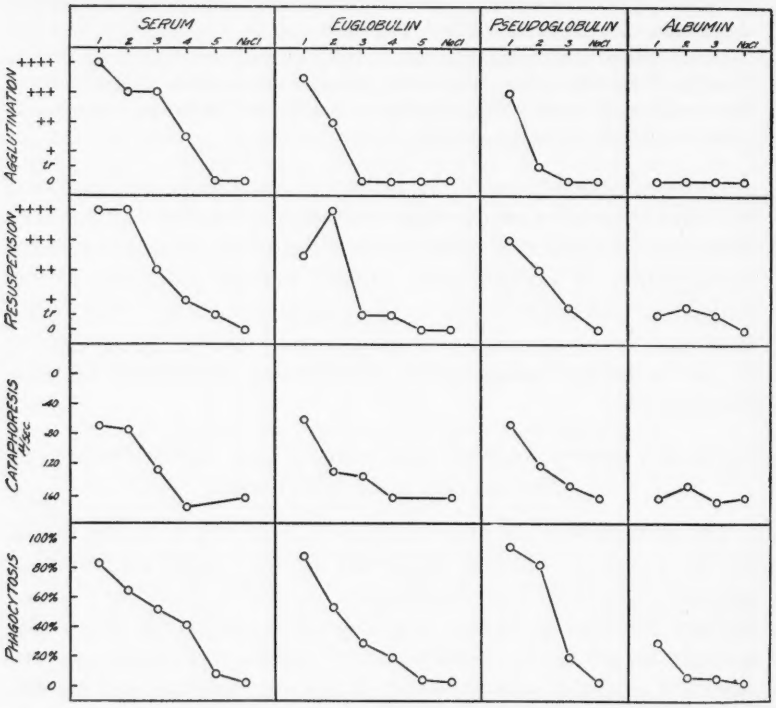

FIG. 1. Parallelism between surface changes and phagocytosis in presence of immune serum and its globulin fractions. Abscissae are successive dilutions of antiserum or serum fraction in powers of four. (Thus 3 is a dilution of $1:4^3$ or 1:64.) *M. avium* (Arloing strain) and homologous antiserum.

experiment of this character in which the tests cannot be perfectly precise and necessarily include in each case variables not common to the other tests. With these slight reservations in mind the impressive fact has been the close correspondence between surface, agglutinating and tropin effects when whole serum or its euglobulin or pseudoglobulin fractions have been used.

In contrast to this were the results when the albumin fractions of antisera were used. The presence of serum albumin in the phagocytic mixture usually induced a moderate degree of phagocytosis. This effect was moderate at a dilution of albumin of 1 to 4, was weaker or

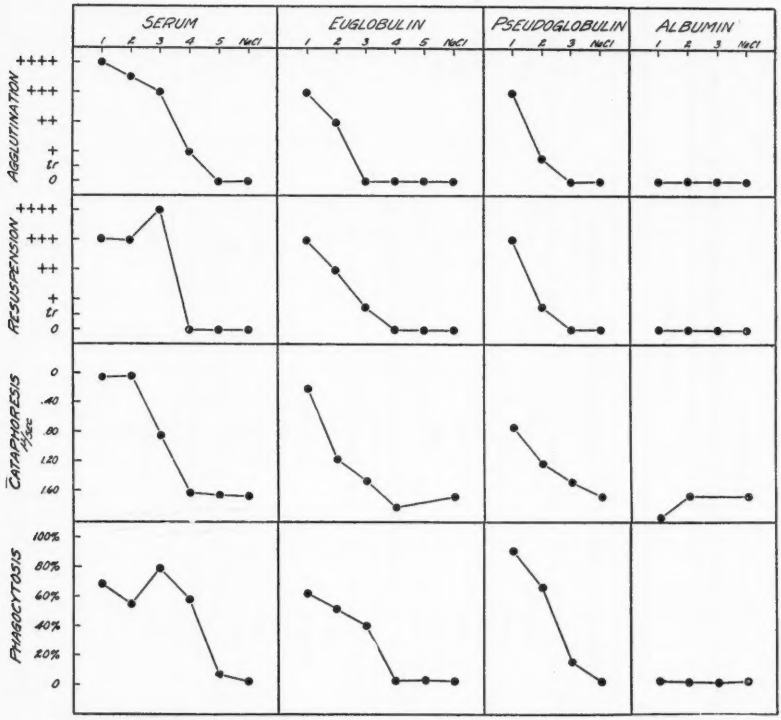

FIG. 2. Parallelism between surface changes and phagocytosis after sensitization with immune serum and its globulin fractions. The same antigen and antiserum as in Fig. 1. The antigen was sensitized and washed, then exposed to phagocytosis.

absent at dilutions of 1 to 16 and 1 to 64, and did not extend to higher dilutions. Serum albumin on the contrary caused only very slight and irregular surface or agglutinating effects and these were essentially out of correspondence with the phagocytosis-promoting effect.

TABLE I

The Effect of Washing upon the Phagocytosis of Bacteria Sensitized with the Globulin and Albumin Fractions of an Immune Serum

	Serum dilutions				NaCl contro
	1:4	1:16	1:64	1:256	
Series A: *M. chelonei* in anti-chelonei serum-euglobulin					
Agglutination	++++ to +++	++++ to +++	++++ to +++	++++ to +++	0
Resuspension	+++	+++	−	++	0
Interface	+++	+++ to ++	−	+	0
Cataphoresis, μ/sec	0	0	−	0.19	1.80
Phagocytosis, washed, %	90	97	−	92	4
Phagocytosis, unwashed, %	97	91	97	97	3
Series B: *M. chelonei* in anti-chelonei serum-pseudoglobulin					
Agglutination	++++ to +++	++++ to +++	++ to +	0	0
Resuspension	++	+	+	0	0
Interface	++ to +	+ to tr.	±	−	−
Cataphoresis, μ/sec	0	0.61	1.11	1.53	1.98
Phagocytosis, washed, %	100	92	83	8	6
Phagocytosis, unwashed, %	98	87	91	16	3
Series C: *M. chelonei* in anti-chelonei serum-albumin					
Agglutination	0	0	0	−	−
Resuspension	tr.	tr.	0	−	−
Interface	0	−	−	−	−
Cataphoresis, μ/sec	1.61	1.65	1.41	−	−
Phagocytosis, washed, %	8	4	6	−	−
Phagocytosis, unwashed, %	54	7	3	−	−

Series D: *M. tuberculosis* in anti-chelonei serum-euglobulin

Agglutination	+++ to ++	++	++	++ to +	tr.
Resuspension	++ to +	+	+	−	0
Interface	++++ to +++	++ to +	+	tr.	0
Cataphoresis, μ/sec	0	0	0	0.59	1.58
Phagocytosis, washed, %	27	30	11	10	3
Phagocytosis, unwashed, %	56	45	48	45	1

Series E: *M. tuberculosis* in anti-chelonei serum-pseudoglobulin

Agglutination	++	++ to +	+ to tr.	tr.	tr.
Resuspension	tr.	++ to +	++ to +	+	+ to tr.
Interface	+	tr.	−	−	−
Cataphoresis, μ/sec	0	0.32	0.58	1.00	−
Phagocytosis, washed, %	38	15	8	−	2
Phagocytosis, unwashed, %	61	49	25	14	0

Series F: *M. tuberculosis* in anti-chelonei serum-albumin

Agglutination	tr.	tr.	tr.	−	−
Resuspension	++ to +	tr.	0	−	−
Interface	0	−	−	−	−
Cataphoresis, μ/sec	0.85	1.14	1.11	−	−
Phagocytosis, washed, %	9	4	2	−	−
Phagocytosis, unwashed, %	28	11	10	−	−

The Effect of Washing the Sensitized Bacteria

Whether phagocytosis was conducted in the presence of the whole immune sera, or whether the bacteria were sensitized and washed and then exposed to the leucocytes, made very little difference. In an occasional experiment the percentage phagocytosis was slightly less after washing, but in the majority it was not affected. The same was true for the euglobulin and pseudoglobulin fractions. Illustrative experiments are given in Figs. 1 and 2 and Table I.

In contrast to the whole serum and globulin fractions, the phagocytosis-promoting effect of the serum albumin was in all cases much reduced or was abolished by washing. This constitutes a second point of differentiation between the phagocytosis-promoting effect of albumin and the true tropin effects of the globulin fractions. A third difference, that of specificity, is brought out in the paper following this.

The Effect of Heating the Sera before Fractionation

Each of three batches of immune serum was divided into two portions, of which one was heated at 56°C. for 30 minutes. The heated and unheated portions of each serum were fractionated and tested with the homologous bacterial strain.

The surface and tropin effects of an anti-avian (Arloing) and of an antichelonei serum were only very slightly if at all weakened by the heating except that, in the case of the antichelonei serum, a definite agglutination and phagocytic prozone appeared in the heated portion; there was no prozone in resuspension, interface or cataphoresis reactions. Such prozones in the other reactions have occasionally been noted in our work with immune sera heated to 56°C. for 30 minutes. They are evidently similar to the more pronounced agglutination prozones produced by Shibley (4) by heating antisera to higher temperatures. The phagocytosis-promoting properties of immune sera may then be impaired by heating, as well as by aging (2), even in instances in which the surface reactions indicate no impairment of the capacity of the serum to combine with antigen.

The surface and tropin effects of the euglobulin fractions of the immune sera were only very slightly if at all weakened by the heating. The surface and tropin effects of the pseudoglobulin fractions of the antisera were not appreciably weakened by the heating.

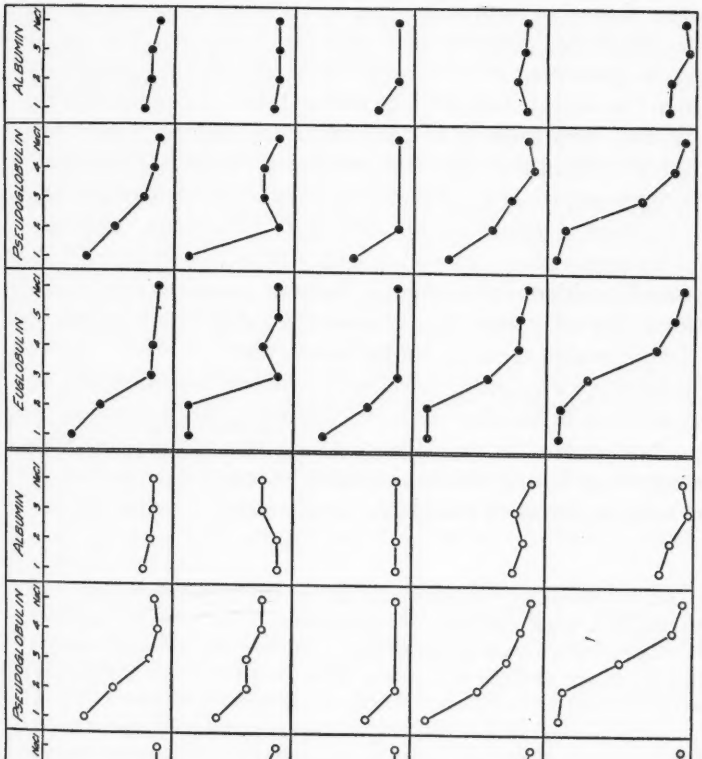

FIG. 3. Parallelism between surface changes and phagocytosis after sensitization with globulin fractions of immune serum. *M. avium* (Arloing strain) sensitized with serum fractions and then washed. Open circles: fractions from Anti-Arloing serum heated for 30 minutes at 56°C. before fractionation. Black circles: fractions from same Anti-Arloing serum unheated.

DISCUSSION

It is clear, then, that the specifically active substances in our immune sera are distributed between the globulin fractions. The small phagocytosis-promoting effect of serum albumin differs in several respects from the tropin action of whole sera and their globulin fractions. The data lead very directly to the conclusion, therefore, that the interaction of antigen and fresh immune serum results in the deposit on the antigen surface of a substance or substances contained in the globulin fractions of the antiserum. The surface reactions, agglutination and increased phagocytosis are quantitatively correlated with this surface deposition, and without a doubt are consequences of the properties of the substances deposited on the antigen surface and of the special environing conditions of the several tests.

The question very naturally arises as to whether this conception of antibody action is in harmony with previous work; this is the case.

It was demonstrated by early investigations that antigens in sensitization acquire a deposit of material which at least has properties in common with the serum of the species from which the antiserum has come.

Ehrlich and Morgenroth (5) demonstrated in 1901 that immune sera produced by a given species, A, when injected into another species, B, led to the development of anti-immune sera which could neutralize the antibodies in immune sera in species A. Bordet (6) showed similarly that normal serum of species A injected into another species produced anti-sera which would neutralize the antibodies in sera of species A. Bordet further showed that such anti-immune sera could even interact with antibodies already united with their antigens and could alter the state of their union with antigen. Thus ox cells sensitized with rabbit immune serum and washed, were "cured" by treatment with anti-sensitizer produced by injection of normal rabbit serum into guinea pigs; addition of complement did not cause hemolysis of the sensitized ox cells. The interaction of sensitizer and anti-sensitizer was shown to be a true combination. Finally it was shown that normal rabbit serum could partially displace rabbit sensitizer from its union with anti-sensitizer.

Bacteria sensitized with immune horse-serum and injected into guinea pigs by Braun (7) made the guinea pigs anaphylactic to horse serum. Bacteria sensitized with immune horse-serum were stated to absorb the anaphylactic antibodies from the serum of guinea pigs and the precipitating antibodies from the sera of rabbits injected with horse serum. Altmann (8), by immunizing animals with sensitized red blood cells, thoroughly washed, obtained sera which gave precipitation and

complement fixation with serum of the species from which the sensitizer had come.

More recent studies by physical-chemical methods have closely associated the sensitizing substance or substances with the globulin of the immune serum.

Porges (9) reported that bacteria treated with a large excess of agglutinin were precipitated by salts in a range of concentration characteristic for serum globulin rather than for the unsensitized bacteria.

Coulter (10, 11) reported that strong sensitization with immune rabbit serum shifted the agglutination optimum of sheep's red blood cells from its value with normal cells of pH 4.75 to a pH of about 5.3; the latter value is close to the isoelectric point of serum globulin. The maximum combination of hemolytic sensitizer from rabbit serum with sheep red blood cells (and minimum dissociation) were stated to occur also at a pH of about 5.3.

The work of Northrop and De Kruif (12), and Northrop and Freund (13) showed that the agglutinating substances from immune sera form a deposit on the surface of the homologous bacteria.

Red blood cells (14), acid-fast bacteria (15) and certain spirochetes (16) have been shown by Mudd and Mudd to have surfaces whose wetting properties are suggestive of a high lipin content. Sensitization of each of these antigens with homologous immune serum gives it an altered surface whose wetting properties are like those of protein.

Shibley (17) found that a variety of bacteria whose surface electric potential differences vary widely, were all brought by sensitization to essentially the same potential difference. The electric behavior of strongly sensitized bacteria closely paralleled that of serum globulin.

Eagle (18) showed that the physical constants for adsorption by sensitized bacteria and red blood cells were closely similar to those for adsorption by immune precipitate. The work of Moll (19), Welsh and Chapman (20), Ottensooser (21), Wu and his associates (22), and Marrack and Smith (23) indicate that a large part of immune precipitate is protein material from the globulin fraction of the antiserum.

Heinemann and Gatewood (24) demonstrated that the bacteriotropins in antistreptococcic and antigonococcic horse sera were precipitated with the globulins.

The work cited thus shows by a number of independent methods that an antigen in specific sensitization receives a surface deposit of a substance or substances which at least have several points of resemblance to serum globulin.

The contributions of Rhumbler (25), Tait (26) and especially those

of Fenn (27) and of Ponder (28) have shown on theoretical grounds that a major requirement for phagocytosis is that the particles to be ingested shall have a surface upon which the leucocytes will spread.

We conclude, therefore, both from our own work and from that of earlier investigators, that *the contact of antigen with fresh homologous immune serum results in the deposit on the antigen surface of a substance or substances contained in the globulin fractions of the antiserum; as a consequence of this surface deposit leucocytes can spread upon and engulf the antigen.*

The conception of antibody action to which we and other students of the physical-chemistry of immune reactions have been led, represents a synthesis of certain elements from the views of the older schools associated, respectively, with the names of Ehrlich and of Bordet. Practically all investigators are agreed, so far as we know, that the combination of antigen and antibody is determined by specific chemical affinities. The beautiful demonstration that the specificity of antigen and antibody may be determined by the spacial configurations about a single carbon atom securely establishes this point of view (29, 30). On the other hand we find no evidence to support the conception of special reacting groups on the antibody responsible for bringing about the several immune reactions. The evidence, at least in the systems with which we have worked, supports the conception of precipitation, agglutination, phagocytosis, and the changes detected in the interface and cataphoresis reactions as consequences, under the several conditions of test, of the properties of the same substances deposited on the antigen surface. The combination of antigen and antibody then is specific; the consequences of that combination are non-specific expressions of certain physical-chemical attributes of the immune substances, and the phenomena exhibited are various rather because of various environing conditions than because of differences in the attributes of the immune substances. Further evidence in support of this conclusion will be presented in the paper following.

We do not wish at the present time to extend this discussion to include lytic or antitoxic reactions. Even in such cases it seems not improbable, however, that the phenomena exhibited may depend more upon the properties of the antigens than upon any special peculiarities of the specific antibodies involved.

SUMMARY

Antisera against several strains of acid-fast bacteria have been separated into their euglobulin, pseudoglobulin and albumin fractions. The globulin fractions have been found to possess the essential properties of bacteriotropic sera: thus they alter the bacterial surface properties, and, in quantitative correspondence, cause agglutination and phagocytosis; these several effects withstand washing of the sensitized bacteria; the effects are little if at all affected by inactivation of the antisera before fractionation; the combination of antibody and antigen is serologically specific.

The conclusion is drawn that the contact of antigen with fresh homologous immune serum results in the deposit on the antigen surface of a substance or substances contained in the globulin fractions of the antiserum; as a consequence of this surface deposit leucocytes can spread upon and engulf the antigen.

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ON THE MECHANISM OF OPSONIN AND BACTERIOTROPIN ACTION

VI. Agglutination and Tropin Action by Precipitin Sera. Characterization of the Sensitized Surface

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In the preceding communication we have advanced a theory of tropin action and offered in its support evidence that we feel to be convincing. The effectiveness of immune sera in agglutinating, altering the surface properties and promoting the phagocytosis of antigens has been found to be dependent upon the presence of substances in the globulin fractions of the serum which combine with and form a deposit on the surface of the antigen. Phagocytosis, the characteristic behavior of the sensitized antigens in resuspension, interface and cataphoresis tests, and agglutination follow essentially as consequences of the properties of the substances deposited on the antigen surface.

In the present communication we shall describe building up artificial surfaces and obtaining agglutination, characteristic surface properties and phagocytosis in accordance with prediction from the theory. In this phase we are following and extending the work of F. S. Jones (1, 2). We find with Jones that precipitin sera will cause the agglutination and phagocytosis of collodion particles previously treated with homologous precipitinogen. The active substances are found both in the euglobulin and pseudoglobulin fractions of the antisera. Precipitation, agglutination, altered cataphoretic properties and phagocytosis are brought about in approximately corresponding degree. And finally the properties of maximally sensitized precipitinogen-treated collodion particles, of specific immune precipitate, and of maximally sensitized acid-fast bacteria are, within the limits of our methods, identical.

Thus we have powerful evidence in support of a rational conception of these diverse serological phenomena as all special consequences of one underlying phenomenon. The combination of antigen and antibody is determined by specific chemical affinities. The effects following this combination, at least with the antigens we have thus far studied, namely precipitation, agglutination, changes in surface properties and phagocytosis are consequences primarily of the properties of the antibody-protein combined with and deposited upon the antigen surface. The actual phenomena exhibited as a consequence of the deposit of the antibody-protein on the antigen surface of course depend also upon accessory factors such as electrolytes, temperature, the presence of leucocytes and other environing conditions.

Experimental Methods

Immune Sera.—Rabbits were injected five times usually at 5-day intervals with solutions of the antigen. These were crystalline egg albumin,* edestin,* casein (Hammarsten), human serum and horse serum. The egg albumin and human serum and their antisera were most satisfactory.

Preparation of Precipitinogen-Treated Collodion.—Loeb (3) showed that protein could be adsorbed on the surface of collodion particles. Brilliant application of this fact to serology was made by F. S. Jones (1, 2).** We have followed essentially the technique of Jones. Spherical collodion particles of about 1 to 2μ in diameter were furnished us through the kindness of Dr. M. Kunitz. One volume of a dense suspension of these particles was allowed to stand usually for several hours in contact with one volume of human or horse serum or of a saturated solution of the protein. The collodion-precipitinogen mixture, after sufficient contact, was centrifugalized, the supernatant fluid decanted and the sediment resuspended in excess of saline. The suspension was again centrifugalized, the supernatant fluid decanted and the sediment resuspended in 0.85 per cent saline.

Tests.—The precipitinogen-treated, washed collodion particles were set up with serial dilutions of the precipitin sera or of the globulin or albumin fractions of these sera. Agglutination was read at short intervals and often again after keeping the tubes overnight in the ice box. Resuspension was often omitted, but was otherwise done according to the usual technique (5). The interface reaction could not be done since the collodion particles themselves are not wetted by oil.

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* For the egg albumin we are indebted to Dr. J. Freund, and for the edestin to Professor D. Wright Wilson.

** Specific agglutination of protein-treated collodion particles was demonstrated independently by Freund (4).

Cataphoresis was carried out as previously described(5).* In the earlier experiments the test particles were suspended in 0.85 per cent sodium chloride solution. In later work the particles were suspended in M/50 acetate buffer of pH 5.2. When isoelectric point determinations were made the particles were suspended in two or more buffers whose reactions were graduated in intervals of 0.4 pH. Buffers were found such that the test particles moved toward the anode in one, toward the cathode in the next in series. The isoelectric point was then estimated by interpolation.

The phagocytosis tests, staining and counting were as previously described (5). The use of essentially the same methods of detecting phagocytosis of collodion particles as had been used with acid-fast bacteria was made possible by the earlier observation of Freund (6) that collodion particles are acid-fast by the ordinary Ziehl-Neelsen technique.

Precipitin tests were set up by adding 0.2 cc. portions of the precipitin serum or serum fraction to 1 cc. each of the serial dilutions of the precipitinogen solution.

Fractionation of the Precipitin Sera.—Fractionation was carried out as previously described (7).

Agglutinin and Tropin Action by Precipitin Sera; Further Verification of the Theory of Tropin Action

Precipitation tests with the dissolved proteins, and agglutination, cataphoresis, and phagocytosis tests with the proteins adsorbed on collodion particles were performed as routine with all of the antiserum fractions and often also with the whole antisera. In the majority of experiments two antigens and their antisera were included and both homologous and heterologous combinations were tested. The data were finally analyzed with reference to certain questions designed, like those in the preceding paper, to test deductions from our theory of tropin action. In addition to verifying the theory the data have yielded strong support of the unitarian hypothesis of antibody action (8).

* A Zeiss water-immersion lens of 0.75 n.a. and 1.9 mm. working distance has been found to be in certain respects superior to the objective previously described. The water-immersion feature is a nuisance; however, for studying particles which settle out quickly, this may be more than compensated for by the advantages of more critical focus and the greater magnification afforded by the water-immersion lens.

The questions and the results of analyses of the data are given below. Details have for the most part been omitted.

I. When precipitinogen-treated collodion particles are sensitized with immune serum or one of its fractions, what is the order of relative effectiveness in (1) phagocytosis, (2) agglutination, (3) cataphoresis, (4) precipitation?

Whole serum was found generally to be more effective in bringing about all of the reactions than either of its globulin fractions; both globulin fractions were strikingly more effective in all of the reactions than the albumin fraction. In precipitation and phagocytosis the euglobulin and pseudoglobulin fractions were about equally effective; in agglutination pseudoglobulin was oftener more effective than euglobulin, in cataphoresis euglobulin was oftener more effective than pseudoglobulin. With the exception of this slight discrepancy in the relation of the euglobulin and pseudoglobulin fractions, therefore, the sera and fractions were effective in the same order in all of the reactions.

It has been supposed that the euglobulin fraction contains all the precipitin (9); certainly in our work we have found it fairly equally distributed between the euglobulin and pseudoglobulin fractions. Possibly it is not irrelevant to the perennial controversy over the distribution of antibodies in the globulin fractions to point out that two of the chief students of globulin chemistry, Chick (10) and Sørensen (11), believe that a clean-cut separation of euglobulin and pseudoglobulin is impracticable. According to Sørensen, "neither fractionation nor dialysis nor a combination of the two methods results in a complete separation of the two globulins." He believes that the euglobulin and pseudoglobulin are associated as a labile compound E_pP_q.

II. When precipitinogen-treated collodion particles are sensitized with immune serum or one of its fractions, are the effects in promoting phagocytosis approximately proportional to the effects on 1) agglutination, 2) cataphoresis, and 3) precipitation?

The correspondence between tropin, agglutinating, cataphoretic and precipitating effects with whole serum and with both globulin fractions was excellent in practically all experiments. There was not good correspondence between the several tests when the serum albumin fraction was used. The results are thus precisely similar to those obtained with acid-fast bacteria (7).

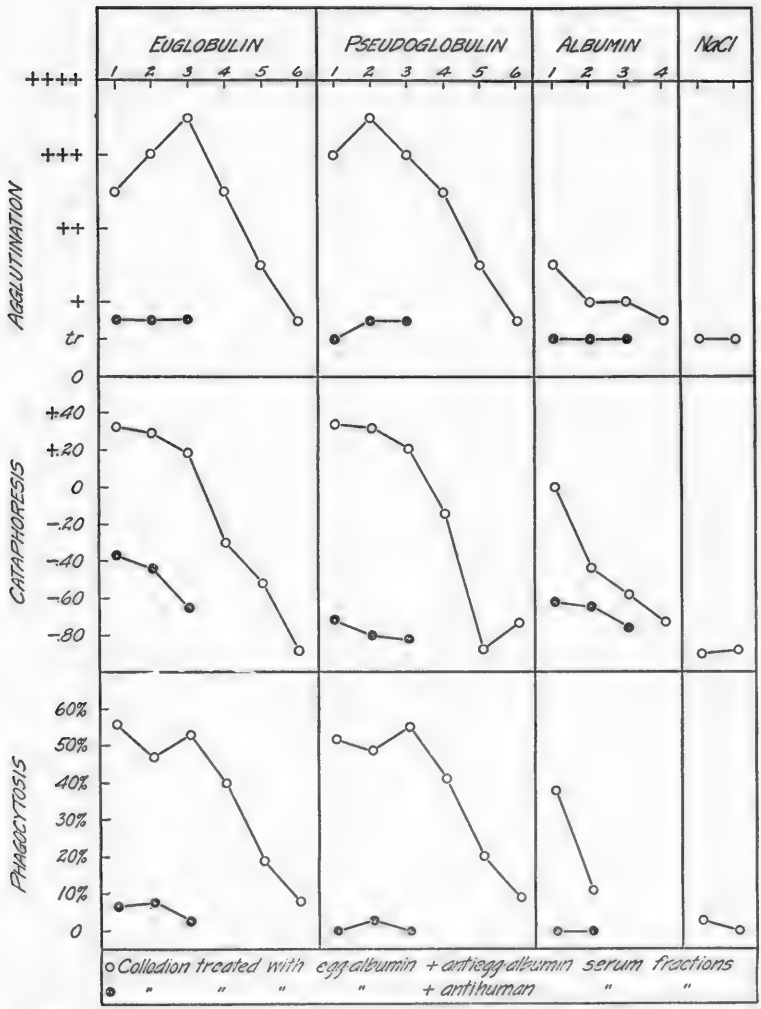

FIG. 1. Parallelism between agglutination, cataphoresis and phagocytosis of precipitinogen-treated collodion particles after sensitization with globulin fractions of precipitin sera. Abscissae are successive dilutions of antiserum or antiserum fraction in powers of four. (Thus 4 is a dilution of $1:4^4$ or 1:256.) All particles were washed after sensitization. Cataphoresis was conducted in M/50 acetate buffer of pH 5.2; graphed as μ/sec. per volt/cm. Note specificity of the reactions.

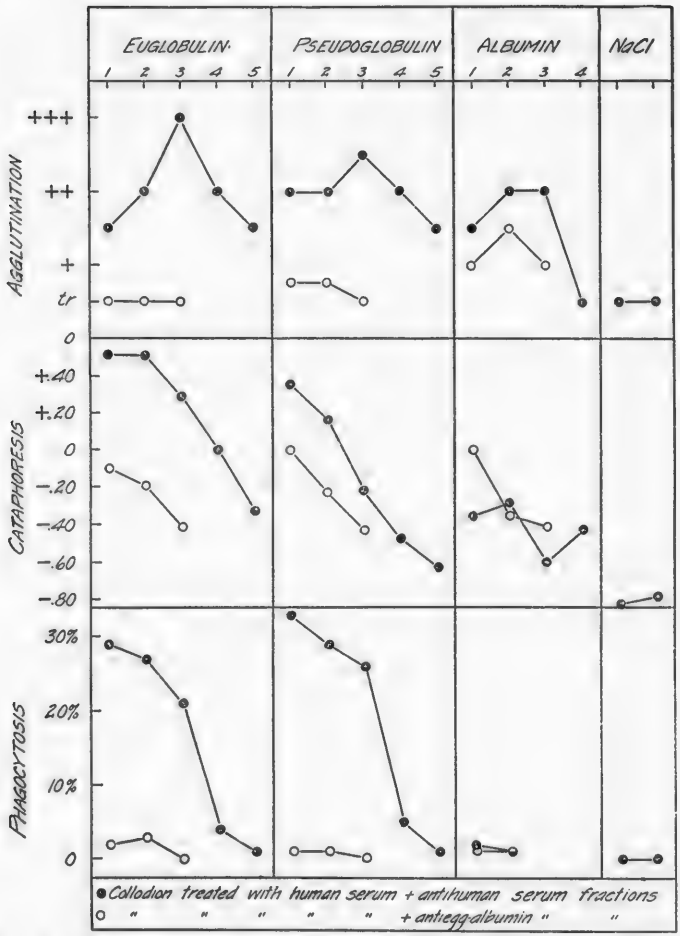

FIG. 2. General correspondence between agglutination, cataphoresis and phagocytosis of precipitinogen-treated collodion particles after sensitization with globulin fractions of precipitin sera. Antisera the same as in Fig. 1. All particles washed after sensitization. Cataphoresis as in Fig. 1. The agglutination prozones are not paralleled in the other reactions.

III. When precipitinogen-treated collodion particles are sensitized with an immune serum fraction is there more interaction with homologous than with heterologous antigen, *i.e.*, are the reactions specific?

The reactions were clearly specific with whole serum, with euglobulin and with pseudoglobulin. This was not the case with serum albumin.

Agglutination by the albumin fraction was greater with homologous than with heterologous antigen in four cases, equal with homologous and heterologous antigens in five. The effect of albumin on cataphoresis was greater with homologous antigen in six cases, greater with heterologous antigen in three cases. The phagocytosis-promoting effect of albumin was greater with homologous antigen in two cases, with heterologous antigen in two cases, and equal with homologous and heterologous antigens in one case; phagocytosis was absent in four cases in which the particles were washed after treatment with serum albumin. It is possible that a trace of specific antibody may have been carried over into the albumin fractions in some experiments and may account for the slightly greater average effect of the albumin fraction with homologous antigen in the agglutination and cataphoresis tests.

IV. What is the effect of washing precipitinogen-treated collodion particles after sensitization with whole homologous antiserum or with one of the several fractions?

The phagocytosis of precipitinogen-treated collodion particles sensitized by whole homologous immune serum or a globulin fraction and then washed proceeded virtually unimpaired. As with the acid-fast bacteria the deposit on the particle surface produced by immune serum or its globulin fractions was a sufficient cause for phagocytosis even in the absence of dissolved tropin.

The phagocytosis found in the presence of the albumin fraction, on the contrary, was much reduced or abolished by washing, as was also the case with acid-fast bacteria. Lack of specificity, lack of correspondence with the other reactions and reduction by washing, therefore, distinguish this phagocytosis-promoting effect of albumin from true tropin action.

Characterization of the Sensitized Surface

The isoelectric point of a protein or other amphoteric substance is a property dependent upon and to a considerable degree characteristic of its chemical structure. The term was originally used in an empiri-

cal sense by Hardy (12) as the reaction at which no migration of the test substance occurs in an electric field. The isoelectric point for the case of a pure amphoteric substance later received mathematical definition in terms of the acid and basic dissociation constants of the substance (13). We shall, however, use the term "isoelectric point" in the original sense of Hardy.

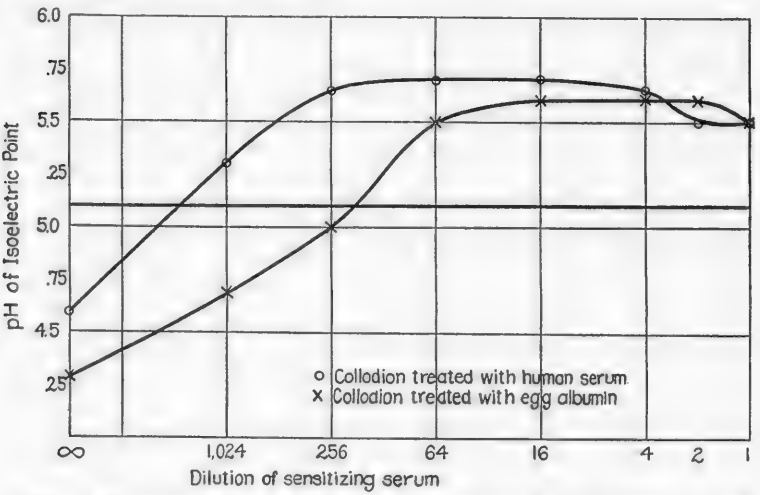

FIG. 3. Progressive change of the isoelectric points of precipitinogen-treated collodion particles with sensitization by homologous precipitin sera. The isoelectric points of the protein-treated collodion particles are about pH 4.3 and 4.6 respectively. After exposure to homologous immune serum in progressively increasing concentrations, the isoelectric points shift to plateau values of pH 5.6 and 5.7. There is a slight prozone with the highest serum concentrations. The horizontal line at pH 5.1 is the mean value for eleven determinations of the isoelectric point of serum euglobulin.

Collodion particles show a strong negative charge in the electric field. When treated with protein by the technique we have followed the particles are isoelectric at a reaction somewhat on the acid side of the isoelectric point of the protein used. This indicates that protein has been adsorbed on the surface of the collodion. The fact that the isoelectric point is on the acid side of that of the protein indicates that the charge is a resultant of that associated with the collodion and that

due to the protein. This supports the conclusion reached by Jones (2) on other grounds that the protein under such circumstances does not form a complete film on the particle.

When such protein-treated collodion particles are sensitized by strong precipitin sera homologous with the protein used, the isoelectric point is shifted to the alkaline side. The shift is progressively greater with progressing concentrations of sensitizing serum until a plateau value of

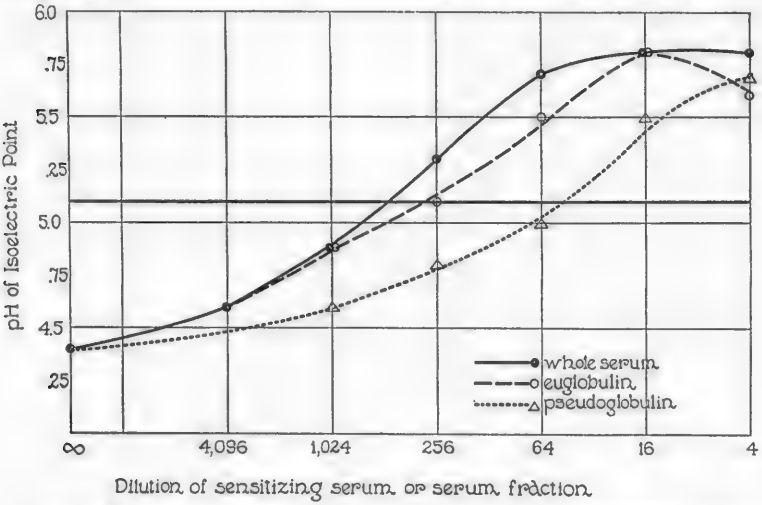

FIG. 4. Progressive change of the isoelectric points of human-serum-treated collodion particles with sensitization by homologous precipitin serum and its globulin fractions. The globulin fractions as well as the whole serum bring the isoelectric point of the antigen within the zone of pH 5.5 to 5.8. Figs. 1, 2, 4 and 5 and Table I all represent data from the same experiment.

pH 5.6 to 5.8 is reached. This effect is seen in Fig. 3. With sera of lower titer greater concentrations of sensitizing serum are required to bring the isoelectric point of the sensitized particle to the plateau value, as seen in Figs. 4 and 5. These figures show that a similar effect is produced by the euglobulin and pseudoglobulin fractions of the immune sera, but that greater concentrations are required to bring it about than with the whole serum. With still weaker immune sera the maximum value reached by the isoelectric point may be below pH 5.6.

The isoelectric points of acid-fast bacteria have been shown in another place (14) to undergo a similar change with serum sensitization. The isoelectric points of the unsensitized bacteria studied were exceedingly low, below pH 2.5; with sensitization by increasing strengths of homologous antiserum the values of the isoelectric points shifted to the alkaline side until values of pH 5.5 to 5.8 were reached.

The isoelectric points of specific precipitates obtained by the interaction of the dissolved proteins and their homologous precipitin sera have

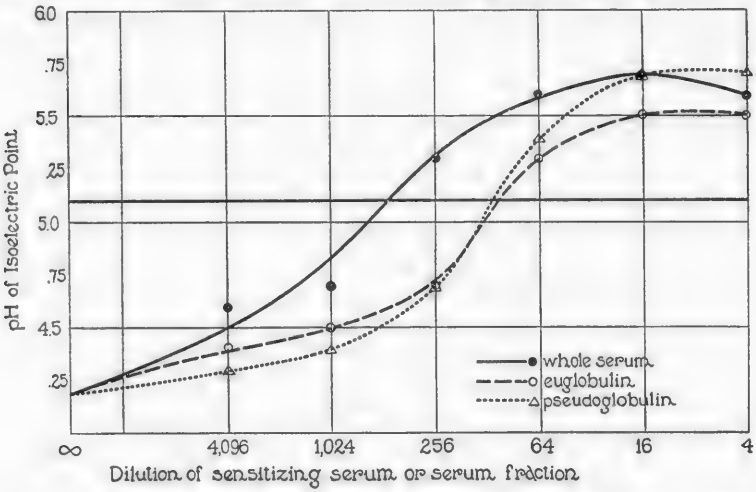

FIG. 5. Progressive change of the isoelectric points of egg-albumin-treated collodion particles with sensitization by homologous precipitin serum and its globulin fractions. The globulin fractions as well as the whole serum bring the isoelectric point of the antigen within the zone of pH 5.5 to 5.8.

also been studied. The results are given in Tables I, II and III. It is evident that the precipitates obtained in the zone of approximately optimal proportions of antigen and antibody are isoelectric in the same range of hydrogen ion concentrations as are maximally sensitized acid-fast bacteria or precipitinogen-treated collodion particles, namely pH 5.5 to 5.8.

With excess of either antigen or antibody the isoelectric points of the immune precipitates have tended to be at slightly lower pH

values.* An analogous instance in which sensitization with the highest concentrations of a high titer immune serum has resulted in a reduction of the isoelectric point to slightly below the maximum value

TABLE I

Isoelectric Points of Specific Precipitates

Immune serum	Dilution of antigen	Precipitate, amount	Isoelectric point of precipitate, unwashed, pH	Isoelectric point of precipitate, washed, pH
Antiegg albumin.....	1:10	+++ to ++	5.15	5.5
" "	1:100	+++	5.35	5.55
" "	1:1000	+++	5.4	5.75
" "	1:10,000	+	—	—
" "	1:100,000	tr.	—	—
Euglobulin from the above serum		—	5.0	5.0
Antihuman serum.....	1:10	+++ to ++	5.4	5.55
" "	1:100	++++ to +++	5.6	5.75
" "	1:1000	++++ to +++	5.4	5.7
" "	1:10,000	++ to +	—	—
" "	1:100,000	+	—	—
Euglobulin from the above serum		—	4.8	4.95

1 cc. each of progressive dilutions of egg albumin solution and of human serum were mixed with 0.2 cc. each of their respective homologous precipitin antisera. Euglobulin was obtained from the same precipitin sera by dilution and acidification. The precipitates were first concentrated by centrifugalization (unwashed); they were then suspended in distilled water, centrifugalized, the supernatant decanted and the sediment resuspended in distilled water (washed); a drop or two of the suspensions were added to several cubic centimeters of dilute acetate or phosphate buffer for the isoelectric point determinations.

is given in Fig. 3. Similar instances have occurred a sufficient number of times in as yet unpublished experiments with potent antisera so

* Incidentally these results show that the often-cited analogy between the zone phenomena in specific precipitation and that seen in the reciprocal precipitation of an electronegative and electropositive colloid is both superficial and misleading. The antigen, the precipitate, and, as far as present evidence shows, the antibody are all electronegative at the hydrogen ion concentration of blood serum.

TABLE II

Isoelectric Points of Specific Precipitates

Antiserum	Dilution of antigen	Precipitate, amount	Isoelectric point of precipitate, pH
Whole antihuman serum	1:10	++	5.4
" " "	1:100	++++	5.7
" " "	1:1000	+++	5.4
" " "	1:10,000	++ to +	—
" " "	1:100,000	tr.	—
Antihuman serum euglobulin	1:10	++	—
" " "	1:100	+++	5.3
" " "	1:1000	+++	5.5
" " "	1:10,000	++	—
" " "	Euglobulin control	+	—
Euglobulin obtained from the above serum by dilution and acidification		—	5.0

1 cc. each of progressive dilutions of human serum were mixed with 0.2 cc. each of whole antihuman precipitin serum or of the euglobulin fraction of this precipitin serum. Euglobulin was obtained from the same precipitin serum by dilution and acidification. The precipitates were washed and isoelectric points were determined as described under Table I.

TABLE III

Isoelectric Points of Specific Precipitates

Dilution of antigen	Precipitate, amount	Isoelectric point of precipitate, pH
1:10	+++	5.4
1:100	++++	5.65
1:1000	+++	5.7
1:10,000	+	5.45
—	Euglobulin	5.25

2 cc. each of progressive dilutions of an egg albumin solution were mixed with 0.4 cc. each of antiegg-albumin precipitin serum. Euglobulin was obtained from the same precipitin serum by dilution and acidification. The immune precipitate and the euglobulin were washed in distilled water; their isoelectric points were determined in dilute acetate buffers.

that it seems unlikely that they can be dismissed as accidental. Heidelberger and Kendall (15) have recently successfully treated the precipitin reaction between Pneumococcus III specific carbohydrate and its corresponding antibody in terms of simple mass action equations. It seems probable that in our experiments the rise of the isoelectric points through a maximum with increasing relative concentrations of antibody may correspond to the transition from the compounds AS_3, through AS_2 to AS as described by Heidelberger and Kendall. Study should be directed explicitly to this point.

TABLE IV

Acid-fast bacteria + homologous antisera or their globulin fractions, result in	altered surface* agglutination phagocytosis
Protein-treated collodion + homologous antisera or their globulin fractions, result in	altered surface* agglutination phagocytosis
Protein solutions + homologous antisera or their globulin fractions, result in	immune precipitate*
Sheep erythrocytes + homologous antisera, result in	altered surface* agglutination phagocytosis

* Properties of altered surface and of immune precipitate:

1. Cohesion high.
2. Wetting properties characteristic of protein.
3. Isoelectric point at pH 5.5 to 5.8.

The wetting properties of immune precipitate when examined in an oil-water interface are also similar to those of maximally sensitized acid-fast bacteria. These properties are characteristic* for protein; for instance globulin, egg-albumin, casein or edestin particles examined in such interfaces exhibit wetting properties similar to those of the immune precipitate or the maximally sensitized bacterial surface.

The analogy between immune precipitate and the maximally sensi-

* Although characteristic, no claim is made that these wetting properties are specific for protein.

tized surface of particulate antigen is rendered virtually complete by the evidence brought forward by Cromwell and Centeno (16) to indicate that specific precipitate is phagocytized.

The chief results of the interaction of antigen and antibody in the systems we have studied are summarized in Table IV.

Earlier work on the isoelectric points of sensitized antigens is reviewed elsewhere (14). The work which has hitherto advanced farthest in characterizing the properties of the sensitized surface, that of Shibley (17), seemed to show that the surface was coated with a film of denatured serum globulin. That the sensitized surface has several points

TABLE V

Isoelectric Points of Euglobulin Samples

Source	Method of preparation	Isoelectric point, pH
Antiegg-albumin Rabbit 29-19	Ammonium sulfate fractionation	5.2
Anticasein Rabbits 32-97 and 33-72	" " "	5.1
Antiegg-albumin Rabbit 29-19	Dilution and acidification	5.0
Antihuman serum Rabbit 31-77	" " "	4.9
Antihuman serum Rabbit 31-77	" " "	5.0
Antiedestin Rabbits 32-96 and 32-98	" " "	5.05
Antiegg-albumin Rabbit 29-19	" " "	5.2
Antiedestin Rabbit 32-96	" " "	5.2
Antiedestin Rabbit 32-98	" " "	5.2
Antiegg-albumin Rabbit 29-19	" " "	5.1
Antihuman serum Rabbit 31-77	" " "	5.1
Average		5.1

of resemblance to a film of serum globulin has been brought out also by the studies of Coulter (18), of Mudd and Mudd (19) and of Eagle (20). Moreover the sensitizing substance or substances are well known to be associated with the globulin in the fractionation of the serum. However, the familiar fact of possessing specific affinities for homologous antigen constitutes one obvious point of distinction between the antibody and the ordinary serum globulin. A further distinction between the properties of the sensitized surface and the serum globulin is brought out in Tables I to V and Figs. 3 to 5.

In Table V are given the isoelectric points of eleven samples of

euglobulin obtained from the precipitin sera. These were usually precipitated by diluting a small volume of the serum with about fifteen volumes of distilled water and adding a few drops of one-tenth normal hydrochloric acid. Two samples were obtained by ammonium sulfate fractionation. The values of the isoelectric points are seen to be in all cases within a range from pH 4.9 to 5.2, with an average of 5.1. The average value is plotted as a horizontal line at pH 5.1 in Figs. 3 to 5. It is thus evident that the isoelectric points of maximally sensitized acid-fast bacteria and precipitinogen-coated collodion particles and also of immune precipitate lie well to the alkaline side of the range of values for the isoelectric points of euglobulin samples precipitated as described from the same antisera.

A greater difference in the same direction has been reported by Felton (21) between the minimum solubilities of antibody-protein and the non-specific globulin of antipneumococcus horse sera. The minimum solubilities of Felton's antibodies lie between pH 6.6 and 6.8. Several interpretations are possible for the isoelectric values of 5.5 to 5.8 we have found for the maximally sensitized surface and for the specific precipitate. Conceivably these values might represent the values of the isoelectric points of the antibodies of rabbit serum; or these values might be a resultant of the isoelectric points of a more alkaline protein analogous to Felton's with more or less non-specific serum globulin adsorbed upon it; or the uncombined antibody might be isoelectric at a higher or lower pH than 5.5 to 5.8 and obtain these values because of changes involved in the union with antigen. Answers to these questions will have to await further investigation.

Useful conceptions of the nature of antibodies have been proposed by Locke, Main and Hirsch (22) and by Manwaring (23). The denaturization of the antibody-protein when deposited upon the antigen surface has been discussed in references 17, 19 and 24.

Throughout this work we have had capable technical assistance from Mr. H. J. Henderson.

SUMMARY

As a further test of the theory of tropin action proposed in the preceding paper artificial surfaces have been prepared, and have been found to be phagocytized according to prediction from the theory.

Protein was adsorbed on collodion particles according to the technique of F. S. Jones. These particles were then agglutinated and prepared for phagocytosis by the corresponding protein precipitin sera. The precipitating, agglutinating, surface and tropin effects for each serum or serum globulin fraction have been found to be in satisfactory quantitative correspondence. All of these effects were serologically specific; all remained almost unaffected by inactivation of the immune sera for 30 minutes at 56°C. or by washing of the particles after sensitization.

The surfaces of particles maximally sensitized by homologous rabbit immune serum or one of its globulin fractions have shown certain characteristic properties, *i.e.*, they were cohesive, had wetting properties characteristic for protein, and were isoelectric at pH 5.5 to 5.8. The same set of properties were found for immune precipitate in the zone of maximal precipitation. The same properties have also been found for maximally sensitized acid-fast bacteria, and for maximally sensitized sheep erythrocytes.

These results indicate, we believe, that precipitation, agglutination, the surface changes and increased phagocytosis are all consequences of one underlying phenomenon. This phenomenon is the specific chemcal combination with, and deposit on the surface of the antigen of antibody protein. The several serological reactions then follow as consequences of the properties of the sensitized surface and of the special environing conditions.

The antibody is contained in the globulin fractions of immune serum, and appears to be a globulin with physico-chemical differences from normal serum globulin.

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THE BACTERIOSTATIC ACTION OF CERTAIN COMPONENTS OF COMMERCIAL PEPTONES AS AFFECTED BY CONDITIONS OF OXIDATION AND REDUCTION

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When Pneumococcus is seeded into plain broth, it is necessary to use a fairly large inoculum in order to insure the initiation of growth. The size of this inoculum increases with the amount of medium to be seeded; as a rule, 0.01 to 0.1 cc. of a plain broth culture is the minimum seeding to be used for 5 cc. of medium. These inocula contain at least 1,000,000 to 10,000,000 cells. What are the factors which make such a large number of cells necessary? Under what conditions would one single cell grow?

An answer to these questions will probably result in a better understanding of the physiology of the pneumococcus cell. But in addition, the preparation of an "ideal" medium (and no medium is "ideal" which does not allow the growth of a single cell) would be of great value in the study *in vitro* of the bacteriostatic and bactericidal properties of the body fluids of immunized and naturally resistant animals. However, the present work was undertaken to meet a purely practical need. During the past few years, considerable difficulties have often been encountered in the preparation of media suitable for the growth of Pneumococcus. Many lots of broth were found to be unable to support growth even when large inocula were used. Yet it has been observed that when various lots of media are prepared from the same meat infusion, but with the addition of different brands of peptones, these various lots differ in their ability to support growth of Pneumococcus, depending upon the kind of peptone employed; in all cases, growth would occur, even with very small inocula, provided a sufficient amount of neutral cysteine or thioglycollic acid was added. It seems therefore that the inability to support growth depends upon

some bacteriostatic property of the peptone and that this effect may be overcome by the addition of reduced thioacids.

It is known in a general way that the living cell can greatly affect the conditions of its environment. In particular, it may be recalled that pneumococcus cells acting on certain metabolites bring about the formation of actively reducing substances (1, 2). If, however, *very small inocula* be employed, the influence of the reducing action of the bacteria can be considered negligible. Advantage may be taken of this observation in devising an experimental method for the study of those properties of the medium which affect the initiation of growth.

Experimental Methods

Cultures.—All experiments were carried out with a strain of Type II Pneumococcus (D/39/79) and an R culture derived from Type II (D/39/R). Occasionally, control experiments were carried out with strains of Type I and Type III Pneumococcus. The results being always substantially the same, only the data obtained with the Type II strain are presented in this article.

Unless otherwise stated, the inoculum consisted of 0.0001 cc. of a 12-hour culture in plain broth.

Ingredients Used in the Preparation of Media.—The beef infusion was prepared as usual, enriched with 0.03 per cent glucose, adjusted to pH 7.8 and autoclaved.

"Tryptophane broth" is the commercial name of a dehydrated preparation of casein hydrolysed with acid. Solutions of 1 per cent of this dehydrated medium were enriched with 0.03 per cent glucose, adjusted to pH 7.8, and autoclaved.

Peptone solutions employed were of 10 per cent concentration; they were adjusted to pH 7.8 and autoclaved.

The cysteine and thioglycollic acid were autoclaved in neutral 1 per cent solutions and kept under vaseline seal. The results reported in this paper were obtained with thioglycollic acid; no difference could be noticed between the action of the two thiol compounds.

The oxidized thiol compound used was a product obtained by the auto-oxidation of thioglycollic acid (Eastman), in the presence of air, with sodium nitroprusside as a catalyst (in dilute ammonia solution); this product will be referred to as "oxidized thioglycollic acid" although its exact nature is not known since it was not obtained in crystalline form.

The Bacteriostatic Action of Peptones and the Possibility of Correcting It by Means of Reduced Thiol Groups

It has been shown previously (3) that the growth of very small inocula of Pneumococcus in plain broth may occur when cysteine

hydrochloride or thioglycollic acid is added to the medium. Experiment 1 gives the minimum concentration of thioglycollic acid which is necessary for growth to occur.

Experiment 1—Varying amounts of thioglycollic acid were added to different portions of the same lot of plain broth (prepared 1 week previous to use), and tubes containing 5 cc. of these media received inocula varying from 0.1 cc. to 0.0000001 cc. of a plain broth culture of Pneumococcus. The cultures were incubated at 37°C. and final readings made after 48 hours incubation.

The results of Experiment 1 illustrate the fact that, in the plain broth under consideration, an inoculum of 0.1 cc. is necessary to obtain

TABLE I

The Influence of Thioglycollic Acid on the Growth of Small Inocula of Pneumococcus in Plain Broth (Peptone A)

Final concentration of thioglycollic acid (in per cent)	Inoculum (cc. of pneumococcus culture)							
	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}
0	+	−	−	−	−	−	−	−
0.001	+	+	+	−	−	−	−	−
0.002	+	+	+	−	−	−	−	−
0.005	+	+	+	+	+	+	+	−
0.01	+	+	+	+	+	+	+	−
0.02	+	+	+	+	+	+	+	+
0.05	+	+	+	+	+	+	+	−

The signs + and − refer to the presence or absence of growth after 48 hours incubation.

the growth of Pneumococcus. However, growth will occur even if the inoculum be only 0.000001 cc. in the presence of 0.005 per cent thioglycollic acid. It is apparent therefore that plain broth exerts a bacteriostatic effect on Pneumococcus and that a concentration of 0.005 per cent thioglycollic acid can overcome this effect.

The object of the next experiment was to determine whether the minimum concentration of thioglycollic acid required for the growth of small inocula of Pneumococcus would be affected by the kind of peptone used in the preparation of the medium.

Experiment 2.—Media were prepared from the same meat infusion but with different peptones, 2 per cent concentrations being used of either one of the four different commercial preparations of peptone designated as A, B, C, and D. Amounts of 5 cc. of these media were treated with different concentrations of thioglycollic acid, inoculated with 0.0001 cc. of pneumococcus culture and incubated at 37°C. Table II shows the results after 24 hours incubation.

The results of Experiment 2 indicate that the bacteriostatic effect of the broth varies greatly with the different peptones used in the preparation of the medium; this is evidence that a part, at least, of this bacteriostatic power is to be traced to the peptone.

TABLE II

The Effect of Using Different Commercial Peptones on the Growth of Pneumococcus in the Presence of Varying Concentration of Thioglycollic acid

Medium: meat infusion with	Final concentration of thioglycollic acid (in per cent)							
	0	0.0001	0.002	0.004	0.006	0.008	0.01	0.02
Peptone A	−	−	−	−	−	−	−	+
" B	−	−	−	+	+	+	+	+
" C	−	−	−	+	+	+	+	+
" D	−	−	+	+	+	+	+	+

The signs + and − refer to the presence or absence of growth after 24 hours incubation.

The results also suggest that the amount of thioglycollic acid necessary for growth to occur might be used as a measure of the bacteriostatic action of the peptone.

The experiments that follow were all performed with peptone A which appears to have the most pronounced bacteriostatic power.

In order to demonstrate that thioglycollic acid can be used as a measure of the bacteriostatic action of the peptone, an attempt was made to establish a quantitative relationship between the amount of peptone added to the meat infusion and the concentration of thioglycollic acid required to overcome the bacteriostatic action of such media.

Experiment 3.—Media were prepared from the same meat infusion, but with concentrations of peptone (brand A) varying from 0.5 per cent to 4 per cent; these media were treated with varying concentrations of thioglycollic acid, inoculated

with 0.0001 cc. of pneumococcus culture (per 5 cc. of medium) and incubated at 37°C.

The results of Experiment 3 indicate that as the concentration of the peptone in the medium increases, the concentration of thioglycollic acid necessary for growth also increases. A more exact relationship will be obtained when, instead of the whole peptone, we use the isolated fraction responsible for the bacteriostatic action (see Experiment 8). This indicates that thioglycollic acid can be used to titrate the bacteriostatic power of the peptone.

TABLE III

The Relation between the Concentration of Peptone (Brand A) Used in the Preparation of the Broth, and the Concentration of Thioglycollic Acid Required for the Growth of Small Inocula of Pneumococcus

Concentration of peptone A (in per cent)	Final concentration of thioglycollic acid (in per cent)						
	0	0.001	0.002	0.004	0.006	0.008	0.01
4	−	−	−	−	−	−	+
2	−	−	−	−	+	+	+
1	−	−	−	+	+	+	+
0.5	−	+	+	+	+	+	+

The signs + and − refer to the presence and absence of growth after 48 hours incubation.

It has been suggested previously (3) that cysteine owes its beneficial action on the growth of Pneumococcus to its reducing power.

This suggestion will be confirmed by comparing the effect of the oxidized and reduced forms of thioglycollic acid on the growth of Pneumococcus in plain broth.

Experiment 4.—Different portions of plain broth prepared with 1 per cent of peptone A were treated with "reduced" and "oxidized thioglycollic acid." Amounts of 5 cc. of these media were seeded with inocula ranging from 0.1 to 0.00001 cc. of pneumococcus culture and incubated at 37°C.

The results of Experiment 4 indicate that, following oxidation, thioglycollic acid becomes unable to correct the bacteriostatic effect of the peptone; it may be noted, however, that "oxidized thioglycollic acid" is in no way toxic, as is shown by comparison with the control.

Thioglycollic acid therefore appears to owe its activity to the presence of reduced thiol groups in the molecule.

The Existence in Peptone A of Substances Which Are Bacteriostatic under Oxidizing Conditions and Not under Reducing Conditions

It has just been shown that the bacteriostatic power of peptone can be corrected by treatment with reduced thiol groups. It is also greatly affected by the conditions of oxidation and reduction under which the peptone solutions are kept. This can be shown by the five following examples.

Experiment 5.—a, b, c. Test tubes containing 4.5 cc. of meat infusion received 0.5 cc. of a 10 per cent peptone solution (at pH 7.8) which had been autoclaved.

In preparation *a*, the peptone solution was used *immediately after autoclaving*; in preparation *b*, it had been kept for 48 hours at room temperature under *aerobic conditions*; in preparation *c* it had been kept for 48 hours at room temperature under *vaseline seal.*

d. The peptone solution which had been kept aerobically for 48 hours (preparation *b*) was added to meat infusion and the mixture kept under vaseline seal for 2 weeks at room temperature.

e. A similar mixture (preparation *d*) which had been kept under vaseline seal for 2 weeks was now transferred to a flask and kept aerobically for 2 days.

Test tubes containing 5 cc. of the media (prepared as described under *a, b, c, d,* and *e*) were inoculated with 0.0001 cc. of pneumococcus culture and incubated at 37°C.

The results of Experiment 5 prove that peptone A used immediately after autoclaving, (preparation *a*), or kept constantly under anaerobic conditions (preparation *c*), exhibits only a very slight bacteriostatic power. On the other hand, the same peptone solution becomes markedly bacteriostatic following exposure to the air (preparation *b*). A peptone solution that has become bacteriostatic following exposure to the air loses its bacteriostatic effect by incubation under vaseline seal in the presence of meat infusion (preparation *d*); but again becomes bacteriostatic following reexposure to the air.

These facts, as well as the beneficial action of reduced thiol groups, point to the existence in peptone A of a substance which is bacteriostatic in the oxidized form, and not in the reduced form; it can be oxidized by atmospheric oxygen and reduced by -SH groups and meat infusion; this oxidation and reduction appears to be reversible.

In the course of these experiments, it was observed that the color of the peptone A varies with the different conditions under which the solution is kept. The solution is dark brown when taken out of the autoclave or when kept under vaseline seal. The color turns yellow

TABLE IV

The Growth of Pneumococcus in Plain Broth (Peptone A) in the Presence of Reduced and Oxidized Thioglycollic Acid

Medium	Inoculum (cc.)				
	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}
Plain broth	+	+	−	−	−
" " + 0.01 per cent reduced thioglycollic acid	+	+	+	+	+
Plain broth + 0.01 per cent oxidized thioglycollic acid	+	+	−	−	−

The signs + and − refer to the presence or absence of growth after 48 hours incubation.

TABLE V

Effect of Maintaining Peptone Solutions under Oxidizing and Reducing Conditions on the Bacteriostatic Effect of Media Prepared with Them

Media*	Final concentration of thioglycollic acid (in per cent)						
	0	0.0005	0.001	0.002	0.003	0.004	0.005
Preparation *a*	−	+	+	+	+	+	+
" *b*	−	−	−	−	−	−	+
" *c*	−	−	+	+	+	+	+
" *d*	−	+	+	+	+	+	+
" *e*	−	−	−	−	−	+	+

* The media referred to as preparations *a*, *b*, *c*, *d*, and *e* are described in the text.

The signs + and − refer to the presence or absence of growth after 48 hours incubation.

on the contrary under aerobic conditions. The yellow color could be changed again to the darker by treatment with thioglycollic acid or cysteine. In other words, the darker color corresponds to the reduced non-bacteriostatic form of the peptone solution, whereas the yellow color corresponds to the oxidized-bacteriostatic form.

It was tempting to suppose that the chromogenic fraction responsible for the change in color was associated with the bacteriostatic agent, and an attempt was made to fractionate peptone A on the basis of this assumption.

Isolation of the Fraction of Peptone A Containing the Bacteriostatic Substance

Different agents were used in an attempt to isolate the chromogenic fractions of peptone A (extraction and precipitation with HCl, NaOH, $(NH_4)_2SO_4$, copper salts, trichloracetic acid, alcohol, acetone, ether). The best results were obtained with the following procedure.

200 gm. of peptone were dissolved in 1000 cc. of tap water and autoclaved. On the following day, the solution was treated with 15 cc. of concentrated hydrochloric acid. A heavy precipitate formed rapidly and was separated by filtration or centrifugalization. This dark green precipitate will be designated as fraction 1.

The filtrate (or supernatant) was made alkaline with 20 cc. of 40 per cent sodium hydroxide and 1000 cc. of acetone was added to the solution. A lighter precipitate of a greyish color separated and was designated as fraction 2.

A further amount of 1050 cc. acetone was added to the supernatant; a dark yellow (resin-like) material was seen to run down the walls of the container and could be separated in a separatory funnel. This will be designated as fraction 3.

After removal of this "yellow fraction," 3 liters of acetone were again added and a heavy white floculum was formed. This will be designated as fraction 4.

The remaining acetone solution was still slightly pigmented yellow; although it was found possible to separate this new pigment in very alkaline solution, no attempt was made to separate it on a large scale. The bulk of the solution was evaporated down and designated as fraction 5.

Each one of the 5 fractions was made up to 2 liters (corresponding therefore to a 10 per cent solution of peptone) and adjusted to pH 7.8. Fraction 1 appeared then as a dark green solution somewhat cloudy. Fraction 2 gave a greyish suspension, little soluble in water, and which settled somewhat on standing. Fraction 3 gave a perfectly clear, but very dark yellow solution. Fraction 4 gave a limpid solution with a very slight yellow tinge (due probably to a contamination with the preceding fraction). Fraction 5 gave a clear, slightly yellow solution.

5 cc. amounts of these different fractions were treated with 0.5 cc. of 1 per cent thioglycollic acid. Within half an hour (at room temperature), fraction 1 began to change color, turning first to a brown-yellow, then to a dark brownish color. The other fractions remained unchanged. It is therefore apparent that fraction 1 is responsible for the change in color observed in the peptone following oxidation and reduction.

It was also observed that following the addition of 0.1 cc. of a 3 per cent solution of potassium cyanide, fraction 1 turned whitish while the other fractions were not affected.

The object of this experiment was to determine which of the fractions were responsible for the bacteriostatic action of peptone A.

Experiment 6.—Media were prepared by the addition of 0.5 cc. of the different fractions and of whole peptone (10 per cent solution) to 4.5 cc. of meat infusion. These media were treated with different concentration of thioglycollic acid and inoculated with 0.0001 cc. of a pneumococcus culture.

TABLE VI

The Bacteriostatic Effect on Pneumococcus of Media Prepared by the Addition to Meat Infusion of Different Fractions of Peptone A

Medium containing	Final concentration of thioglycollic acid (per cent)					
	0	0.001	0.002	0.003	0.004	0.005
Fraction 1	−	−	−	−	−	−
" 2	+	+	+	+	+	+
" 3	+	+	+	+	+	+
" 4	+	+	+	+	+	+
" 5	−	−	−	−	−	−
Whole peptone	−	−	−	−	+	+

The signs + and −refer to the presence or absence of growth after 48 hours incubation.

It is apparent from the results of Experiment 6 that fractions 2, 3 and 4 of peptone A can support the growth of Pneumococcus when added to meat infusion—*and do not exhibit any bacteriostatic effect.* The absence of growth with fractions 1 and 5 may be attributed to the lack of some factor essential to growth or to the bacteriostatic action of these fractions. This question is the subject of the following experiment.

Experiment 7.—A medium was prepared by the addition of 15 cc. of fraction 4 to 135 cc. of meat infusion. To tubes containing 5 cc. amounts of this medium was added 0.5 cc. of peptone solution (10 per cent), of fraction 1 or of fraction 5 along with different concentrations of thioglycollic acid. The tubes were then inoculated with 0.0001 cc. of pneumococcus culture and incubated at 37°C.

The results of Experiment 7 demonstrate that practically all the bacteriostatic action of the peptone is due to fraction 1 and only to it.

In all these experiments, thioglycollic has been used as a reagent for titrating the bacteriostatic action of the materials under consideration. In fact, it has been found in Experiment 3 that the amount of thioglycollic acid required to initiate growth of small inocula increases with the concentration of peptone in the medium. The subject of Experiment 8 was to determine whether a more exact relationship could be obtained between the amount of fraction 1 added to the medium and that of thioglycollic acid required to overcome its bacteriostatic action.

Experiment 8.—Attention must be called to two modifications introduced in the experimental procedure. Meat infusion is known to possess a high reducing

TABLE VII

The Bacteriostatic Action of Fraction 1 of Peptone A

Medium: meat infusion with	Concentration of thioglycollic acid (per cent)						
	0	0.0004	0.002	0.005	0.01	0.02	0.05
Fraction 4	+	+	+	+	+	+	+
" 4 + fraction 1	−	−	−	+	+	+	+
" 4 + fraction 5	−	+	+	+	+	+	+
Whole peptone	−	−	−	+	+	+	+

The signs + and − refer to the presence or absence of growth after 48 hours incubation.

capacity and it is likely that it would thus interfere with the establishment of the quantitative relationship between fraction 1 and thioglycollic acid. Tryptophane broth on the contrary has very little reducing capacity; it can support the growth of Pneumococcus when added to fraction 3 of peptone. It was therefore used instead of meat infusion in this experiment.

The other modification was devised in view of the fact that the fraction 1, after being reduced by thioglycollic acid, may be reoxidized by atmospheric oxygen. This was prevented by incubating the culture under vaseline seal.

Varying amounts of fraction 1 were added to test tubes containing 4.5 cc. of medium consisting of tryptophane broth and fraction 3; these media were treated with different concentrations of thioglycollic acid, inoculated with 0.0001 cc. of pneumococcus culture and incubated under vaseline seal.

Table VIII brings out the close proportionality between the concentration of fraction 1 in the medium and the amount of thioglycollic

TABLE VIII

Titration of the Bacteriostatic Fraction of Peptone A by Means of Thioglycollic Acid

Amount of fraction 1 added to the medium (in per cent)	Final concentration of thioglycollic acid (in per cent)						
	0	0.0005	0.001	0.002	0.005	0.01	0.02
0	+	+	+	+	+	+	+
0.2	−	+	+	+	+	+	+
0.5	−	−	−	−	+	+	+
1.0	−	−	−	−	−	+	+
2 0	−	−	−	−	−	−	+

The signs + and − refer to the presence or absence of growth after 48 hours incubation.

TABLE IX

The Effect of Potassium Cyanide on the Bacteriostatic Properties of the Fraction 1 of Peptone A

Medium: meat infusion with	Final concentration of thioglycollic acid (in per cent)				
	0.0005	0.002	0.005	0.01	0.02
Fraction 4	+	+	+	+	+
KCN + fraction 4	+	+	+	+	+
Fraction 1 + fraction 4	−	−	−	+	+
KCN + fraction 1 + fraction 4	+	+	+	+	+

TABLE X

The Growth of Pneumococcus in Media Prepared with Whole Peptone and "Purified Peptone"

Materials added to meat infusion	Final concentration of thioglycollic acid (in per cent)						
	0	0.0001	0.0002	0.005	0.01	0.02	0.05
Whole peptone	−	−	−	−	−	−	+
Purified peptone	−	+	+	+	+	+	+
Fraction 1	−	−	−	−	−	−	−
Purified peptone + fraction 1	−	−	−	−	−	−	−

The signs + and − refer to the presence or absence of growth after 48 hours incubation.

acid required for growth to develop. This relation, however, does not apply when very small concentrations are used.

It has been mentioned previously that, when treated with potassium cyanide, the green pigment rapidly becomes whitish. Experiment 9 demonstrates that fraction 1 loses at the same time its bacteriostatic properties.

Experiment 9.—A medium was prepared by the addition of 10 per cent of fraction 4 to meat infusion. Test tubes containing 5 cc. of this medium received 0.5 cc. of fraction 1, or 0.02 cc. of 3 per cent KCN, or both. They were then treated with different concentrations of thioglycollic acid, inoculated with 0.0001 cc. of pneumococcus culture and incubated at 37°C.

The results of Experiment 9 show that the bacteriostatic properties of fraction 1 can be removed by treatment with potassium cyanide.

The preceding experiments suggest that a solution of peptone A from which the green bacteriostatic fraction has been removed should supply a satisfactory medium when added to meat infusion.

Experiment 10.—A 10 per cent solution of peptone A was treated with the proper amount of concentrated HCl to precipitate fraction 1. This fraction was then made up to the original volume of the peptone solution and adjusted to pH 7.8.

The peptone solution from which fraction 1 had been removed appeared as a yellow fluid which was also adjusted to pH 7.8. This second fraction will be designated as "purified peptone."

These two solutions were added in amounts of 0.5 cc. to 4.5 cc. of meat infusion as described in Table X and the media treated with different concentrations of thioglycollic acid. The tubes were inoculated as usual with 0.0001 cc. of pneumococcus culture and incubated at 37°C.

The results of Experiment 10 demonstrate that by acid precipitation, one can remove almost completely the bacteriostatic properties of a solution of the commercial peptone A.

DISCUSSION

The facts recorded in this article seem to warrant the conclusion that there exists in commercial peptones a substance (or substances) which may exert a bacteriostatic action on the growth of certain organisms. Its action in plain broth is well demonstrated by the lack

of growth of small inocula of Pneumococcus under ordinary conditions. When, on the contrary, the same medium is prepared with peptones from which the bacteriostatic substance has been removed, or when the bacteriostatic effect has been corrected by reduction, inocula consisting of only one or a very few cells are sufficient to initiate growth.

The bacteriostatic substance is heat stable and, in the brand of peptone which has been studied, it is associated with a certain fraction obtained by acid precipitation; this fraction is pigmented, and changes color upon oxidation and reduction; the bacteriostatic property of the same peptone fraction is also controlled by conditions of oxidation and reduction. But we have no other evidence that the chromogenic substance and the bacteriostatic substance are one and the same thing.

Concerning the mode of action of this substance, we have at least an excellent clue; it is bacteriostatic only under oxidizing conditions, not at all under reducing conditions. In this respect, it behaves similarly to some dyes previously studied (indophenols, methylene blue, gallocyanine,[1] which are bacteriostatic in the oxidized form, but not in the reduced form (3).

Methylene blue has been found to be bacteriostatic for Pneumococcus in concentrations as low as 0.00003 molar. There is as yet no direct way of measuring at what minimum concentration the bacteriostatic substance of peptone exerts its activity, but some conception can be gained by an indirect method. As already shown, there exists a certain quantitative relationship between the amount of bacteriostatic substance and the concentration of thioglycollic acid which can overcome its action. In certain experiments, it has been sufficient to add as little as 0.0003 per cent of this thiol compound to allow the growth of an inoculum which would not have otherwise developed; the molecular weight of thioglycollic acid being 92, one sees that the molar concentration is of the same order as that found in the case of methylene blue. Expressed in other terms, this means that the addition of 0.000003 gm. of thioglycollic acid per cubic centimeter of medium may determine whether growth will take place or not. These concentrations are of the order found in catalytic reactions, and one may well wonder whether the bacteriostatic substance of the peptone

[1] The data concerning the effect of gallocyanine have not yet been published.

does not act as anticatalyst in some phase of the metabolism of the cell.

Whatever the mechanism of its action may be, this substance is active in extremely small amounts. We have evidence of organisms which are much more sensitive to it than the ordinary strains of Pneumococcus. It will be worth while to study the rôle of such substances in determining whether a bacterial species is "aerobic" or "anaerobic," whether it is "easy" or "hard" to grow.

The growth of large inocula of Pneumococcus in plain broth, as compared with the lack of development of smaller inocula, is now readily understood. It has been shown (1, 2) that pneumococcus cells, when in the presence of certain metabolites, give rise to a very actively reducing system; this system is able to reduce (and therefore inactivate) the bacteriostatic substance of the peptone and thus renders the medium suitable for the development of growth.

The significance of the facts presented in this article may well transcend their importance for the growth of Pneumococcus in artificial media. One wonders whether there do not exist in tissues mechanisms similar to the one described, which would render impossible the multiplication of bacteria under normal conditions of oxidation. If, however, local reducing conditions were to develop, is it not possible that bacterial invasion of the tissues would then be facilitated? It is well to remember, in this respect, that the peptones under consideration were probably prepared from materials of animal origin. In fact, tryptic digest broth and peptic digest broth prepared from beef were also found to exhibit some bacteriostatic properties, but this action was demonstrable only under aerobic conditions.

SUMMARY

There are present in commercial peptones substances which exhibit bacteriostatic properties for certain bacterial species. These substances are bacteriostatic in the oxidized form, but not in the reduced form. Their bacteriostatic action can be overcome, and their concentration titrated, by the addition of reduced thiol compounds to the media in which they are present.

Different brands of peptone differ greatly in the amount of bacteriostatic substances they contain; these differences account, in part at

least, for the fact that media prepared from the same meat infusion, but with different kinds of peptone, vary in their ability to support bacterial growth.

The bacteriostatic fraction of a certain peptone solution can be completely removed by precipitation with acid and acetone. A peptone which has thus been purified becomes capable of supporting the growth of very small inocula of Pneumococcus.

The significance of the sensitiveness of certain bacterial species to substances which are bacteriostatic in the oxidized but not in the reduced form is considered with reference to (a) the mechanism of bacteriostasis, (b) the growth of bacterial species in artificial media, (c) the problem of infection.

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EXPERIMENTS ON ANAPHYLAXIS TO AZOPROTEINS
Third Paper

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In preceding papers (1, 2) it has been shown to be possible to induce anaphylaxis in guinea pigs by injecting azoproteins, namely, horse serum coupled with diazotized *p*-arsanilic acid. Animals treated in this way become sensitive to azoproteins containing the same azocomponent but another sort of protein; thus the reactions obviously depend upon the arsanilic acid group.

These results have been confirmed by Meyer and Alexander (3) who used also the method of passive sensitization, and by Klopstock and Selter (4). Some of the observations of these workers will be discussed later. Another investigation on the subject has been made recently by Tillett, Avery and Goebel (5). These authors employed as antigens azoproteins prepared by combining globulin or albumin with diazotized glucosides.

The present study was undertaken with the purpose of investigating the specificity of the anaphylactic reactions and of gaining further information on the inhibition phenomenon produced by simple chemical substances (2). In order to test the specificity of anaphylaxis to azoproteins, antigens were selected which differed only in the steric configuration of the specifically reacting groups. The fact that antigens containing sterically isomeric groups are serologically different has already been shown by means of the precipitin reaction by Landsteiner and van der Scheer (6,7) for *l*- and *d*-phenyl (*p*-aminobenzoylamino) acetic acids and *l*-, *d*-, and *m*-tartaric acids. Similar results were obtained by Avery and Goebel (8, 9) who used as antigens glucosides containing glucose or galactose.

EXPERIMENTAL

Specificity of the Anaphylactic Reaction.—For the following experiments antigens were used containing the radicals of the *d*- and *l*-tartaric acids. These were prepared by coupling horse serum with *d*- and *l*-*p*-aminotartranilic acid in the manner previously described (7).

For the sensitization guinea pigs were injected intraperitoneally three times at weekly intervals with a suspension of 1 cc. of the antigens containing about 5 per cent protein. The animals weighed 210 to 250 gm.; the injections were well tolerated.

The solutions used for the reinjection were prepared in the same manner as the sensitizing antigens except that chicken serum was employed instead of horse serum and that after precipitation with acid, the azoproteins, without treatment with alcohol, were brought in solution with the aid of sodium carbonate; the solutions were made isotonic and adjusted to litmus neutrality. The stock solutions of the antigen were brought to a protein content of 3.5 per cent. The test injections were made intravenously 3 weeks after the last administration of the sensitizing dose, with 1 cc. of various dilutions or a larger volume of the concentrated antigen. At the time of the test the weight of the animals was about 400 gm. The results of an experiment in which the specificity of the reaction was tested are given in Table I.

From Table I it is seen that, with one possible exception, the sensitization succeeded regularly; one animal showed only slight symptoms. The quantity sufficient for inducing shock was as low as 0.35 to 0.7 mg. The symptoms were in all cases typical of anaphylactic shock, and in the animals which died, the lungs were distended and the heart was beating. The reactions were strikingly specific since an injection of about 50 to 100 minimal lethal doses was innocuous for the animals sensitized to the heterologous antigen, apart from a drop in temperature which generally did not exceed 1°. In this respect the results are in full agreement with those reported by Tillett, Avery, and Goebel (5).

A further proof of the specificity of the reactions was furnished by reinjecting, on the following day, some of the animals which had received a dose of the heterologous antigen without showing symptoms of anaphylactic shock. Such animals, with one exception, reacted to

a subsequent injection of the same quantity of the homologous antigen, although there was evidence of some protection (Table II).

TABLE I[1]

Animals Sensitized with d-Antigen

Tested with *d*-antigen				Tested with *l*-antigen			
Guinea pig No.	Quantity of azo-protein injected in mg.	Subsequent change in body temperature	Result, symptoms	Guinea pig No.	Quantity of azo-protein injected in mg.	Subsequent change in body temperature	Result, symptoms
		°C.				°C.	
1	70		† 4 min.	14	70	−0.7	Negative
2	35		† 3 "	15	70	−0.4	"
3	35	−4.3	Severe	16	35	−1.5	Slight
4	35		† 3 min.	17	35	−0.7	Negative
5	35		† 3 "	18	17.5	−0.4	"
6	35		† 3 "	19	8.8	−0.9	"
7	35		† 5 "	20	0.7	+0.9	"
8	17.5	−3.3	Severe				
9	17.5		† 4 min.				
10	8.8		† 3 "				
11	1.5		† 4 "				
12	0.7	−2.3	Very severe				
13	0.35	−0.6	Moderate				

Animals Sensitized with l-Antigen

Tested with *d*-antigen				Tested with *l*-antigen			
Guinea pig No.	Quantity of azo-protein injected in mg.	Subsequent change in body temperature	Result, symptoms	Guinea pig No.	Quantity of azo-protein injected in mg.	Subsequent change in body temperature	Result, symptoms
		°C.				°C.	
21	70	+1.0	Negative	26	70		† 4 min.
22	35	−1.1	"	27	35	−2.8	Slight
23	8.8	−1.9	"	28	35		† 3 min.
24	3.0	−0.5	"	29	8.8		† 3 "
25	0.7	−0.6	"	30	3.0		† 5 "
				31	0.7		† 5 "
				32	0.35	−2.4	Severe
				33	0.18	−1.3	Slight

[1] The designations correspond to those in the previous paper (7).

† Death of animal.

Guinea pigs which were sensitized with only one injection of 1 cc. of the antigen showed the same degree of sensitivity as those in the

TABLE II

Reinjection Experiments

Animals Sensitized with d-Antigen

Guinea pig No.	Quantity of *l*-antigen injected in mg.	Result, symptoms	Quantity of *d*-antigen injected in mg.	Subsequent change in body temperature	Result, symptoms
				°C.	
17	35	Negative	35	−1.8	Negative
18	17.5	"	17.5		† 5 min.
19	8.8	"	8.8	−2.5	Severe
20	0.7	"	0.7		† 8 min.

Animals Sensitized with l-Antigen

Guinea pig No.	Quantity of *d*-antigen injected in mg.	Result, symptoms	Quantity of *l*-antigen injected in mg.	Subsequent change in body temperature	Result, symptoms
				°C.	
21	70	Negative	70		† 8 min.
22	35	"	35		† over night
24	3	"	3	−2.2	Severe
25	0.7	"	0.7		† 5 min.

TABLE III

Guinea pigs weighing 200 to 220 gm. were given one intraperitoneal injection of *d*-antigen: reinjection with shocking antigen at the end of 23 days when the animal weighed about 300 gm.

Guinea pig No.	Quantity of *d*-antigen injected in mg.	Subsequent change in body temperature	Result, symptoms
		°C.	
34	3		† 3 min.
35	0.7		† 4 "
36	0.7		† 5 "
37	0.35	−0.7	Moderate to severe

experiment reported in Table I since they also succumbed to a shocking dose of 0.7 mg. An example is given in Table III.

The above experiments furnished hardly any evidence of the inhibition of the anaphylactic reaction by the administration of large doses of the shocking antigen, which was observed by Klopstock and Selter (4). However there was some indication of the zone phenomenon in experiments (Table IV) in which animals were sensitized with an antigen made by coupling beef serum with diazotized arsanilic acid as was done by Klopstock and Selter.

It is seen from Table IV that only one of three animals was killed

TABLE IV

Eleven guinea pigs were sensitized by one subcutaneous injection of atoxyl beef antigen (1 cc. = 16.6 mg.) which was purified by means of acid and alcohol; the reinjection with atoxyl chicken antigen prepared in the same manner as the beef antigen was made 16 days after the sensitization. The reinjection was made intravenously in a volume of 1 cc.

Guinea pig No.	Quantity of chicken antigen injected in mg.	Subsequent change in body temperature	Result, symptoms
		°C.	
38	6	−6.2	Very sick
39	6	−1.1	Negative
40	6		† 5 min.
41	1.5	−1.8	Slight to moderate
42	1.5		† 4 min.
43	1.5		† 7 "
44	1.5		† 7 "
45	1.5		† 6 "
46	0.5	−1.3	Slight to moderate
47	0.5		† 5 min.
48	0.5		† 6 "

by the largest dose employed while four out of five succumbed to a dose four times smaller.

Sensitization with Antigens Prepared According to the Method of Klopstock and Selter.—Whilst in the first experiments on anaphylaxis to azoproteins, the antigens used for sensitization were isolated after coupling in alkaline solution by precipitation with acid, Klopstock and Selter sensitized guinea pigs by injecting guinea pig serum to which they added neutralized diazosolutions. The reinjections were made with azoproteins prepared from guinea pig serum according to the older

method. In the experiment to be described (Table V) the procedure of Klopstock and Selter was followed.

The results as judged from the reinjection with the chicken serum preparation confirmed in a general way those of Klopstock and Selter except that the sensitization did not succeed regularly. They differed in that most of the animals tested did not react to the guinea pig serum

TABLE V

Eighteen guinea pigs were sensitized by a subcutaneous injection of 1 cc. of a solution made by adding two volumes of 1 per cent neutralized solution of diazotized *p*-arsanilic acid to one volume of fresh guinea pig serum (4). The solution stood overnight in the ice box before injections were made. The reinjections were made after an interval of 33 days.

Reinjection with chicken *p*-arsanilic acid antigen (1 cc. = 18.6 mg. protein)				Reinjection with guinea pig *p*-arsanilic acid antigen (1 cc. = 23.8 mg. protein)			
Guinea pig No.	Quantity of antigen in mg.	Subsequent change in body temperature	Result, symptoms	Guinea pig No.	Quantity of antigen in mg.	Subsequent change in body temperature	Result, symptoms
		°C.				°C.	
49	9.0		† 4 min.	59	12	−1.2	Negative
50	9.0	−1.3	Slight	60	12	−0.4	"
51	5.0		† 3 min.	61	5	−1.5	Slight to moderate
52	5.0		†20 "	62	5		† 4 min.
53	5.0	−0.3	Negative	63	5	−0.2	Negative
54	5.0		†20 min.				
55	5.0	−1.0	Negative				
56	1.5	−0.3	"	64	1.5	−0.6	"
57	1.5		† 6 min.	65	1.5	−1.8	"
58	1.5	−0.8	Negative	66	1.5	−0.6	"

preparation. No attempt was made to inquire into the cause of this discrepancy.

From their observations Klopstock and Selter conclude that for the sensitization and the production of antibodies, as well as for the reactions *in vitro*, it is not necessary to have a chemical combination of the azocomponents with protein but that it suffices to use simple "mixtures" of diazocompounds and protein. They stress the view that the diazocompounds would, by themselves, act as antigens and the proteins only enhance the antigenic activity which is inherent in

the simple substances (4, 10, 11). However, as has been shown by Heidelberger and Kendall (12) and one of the present writers (13), there undoubtedly takes place a combination of the diazocompounds with proteins also in neutral solution under the conditions of the method of Klopstock and Selter. Consequently their procedure does not involve a new principle but must be looked upon as a modification of the older method of preparing azoproteins. It is true that in a footnote to their last publication[1] the authors make a statement which may be interpreted as an admission that they dealt not with mixtures but with chemical combinations. As for those instances in which Klopstock and Selter succeeded in sensitizing with diazocompounds alone, it can be assumed that these substances combined with the proteins of the animal injected, so that in this case also the immunization is probably attributable to an azoprotein. Indeed it has been shown that animals can be immunized by azoproteins the protein part of which is derived from their own species (14).

The phenomenon observed by Klopstock and Selter, that guinea pigs sensitized with diazosolutions alone exhibit skin reactions on intradermal injections of the same diazocompound, may be due to a special mechanism if further study should show that the skin reactions can be induced in this way only and not by sensitization with azoproteins. One may suppose either that the chemical combinations formed in the body on the injection of diazosolutions are different from those prepared *in vitro* or that the sensitization is brought about by the direct action on tissues (skin) by the diazocompound as such. Even in the latter case, because of the ease with which diazosolutions combine with proteins it would not be justifiable to draw conclusions, from the experiments discussed, upon the possibility of sensitization with simple chemical substances in general, particularly those which do not readily form compounds with proteins.

Inhibition of Anaphylactic Shock by Simple Substances.—In the experiments of Landsteiner (2) a peculiar phenomenon was noticed, *i.e.* shock could be prevented by injecting sensitized animals with simple azocompounds containing the same specific group as the sensitizing antigen. The substances used were compounds prepared

[1] See (4), page 465.

by coupling diazotized arsanilic acid with tyrosine or *p*-oxybenzoic acid. Analogous results were described by Klopstock and Selter (4) with the sodium salts of *p*-arsanilic acid (atoxyl) and *m*-aminobenzensulfonic acid. K. Meyer did not succeed in obtaining antianaphylaxis by injecting atoxyl into animals sensitized to azoproteins prepared from *p*-arsanilic acid.

Similar experiments were carried out by Tillett, Avery, and Goebel (5) with guinea pigs sensitized to azoproteins containing glucosides. When these animals were injected with uncombined glucoside immediately prior to the administration of the antigen, shock could be prevented. If, however, the shocking injection was given 2 hours later protection was no longer demonstrable. Consequently the authors raise the question as to the mechanism of this inhibitory effect which, indeed, can hardly be looked upon as a desensitization on account of its transitory nature.

Our present experiments were carried out with a series of animals sensitized with antigens prepared from *d*- and *l*-*p*-aminotartranilic acid and another series sensitized to azoproteins prepared from *p*-arsanilic acid.

Guinea pigs weighing 200 to 250 gm. were sensitized as in the previous experiment (see Table I) and were tested 3 weeks after the last injection. At various intervals before the administration of the shocking homologous antigen the animals were injected with solutions of an azocompound made by coupling resorcinol with diazotized *d*- and *l*-*p*-aminotartranilic acids. The products are designated as *d*-T.R. and *l*-T.R., respectively.

These substances were prepared as follows: 480 mg. *d*- or *l*-*p*-aminotartranilic acid were diazotized in the usual way (7, page 410) and coupled with 110 mg. resorcinol. The dye formed was precipitated with the aid of dilute hydrochloric acid, the precipitate washed in acidulated water and dried.

A 1 per cent solution of the dye was made in a 0.9 per cent salt solution by adding dilute sodium hydroxide, and the solution was adjusted to neutrality or faint alkalinity. 1 cc. of various dilutions was injected intravenously. The shocking antigen was injected in a quantity of 7 mg. (1 cc. of a 0.7 per cent dilution) which corresponds to 5 to 10 minimal lethal doses (see Table I). At the time of the tests the weight of the guinea pigs was about 400 gm.

The experiments (Table VI) demonstrate that with one exception in which the animal showed severe anaphylactic symptoms, the guinea pigs previously injected with the heterologous azodye died in typical

TABLE VI

Inhibition by the Injection of Azodyes

Animals Sensitized with d-Antigen

Guinea pig No.	*d*-T.R. mg.	Interval between injections in hours	Result and symptoms after injection of *d*-antigen	Subsequent change in body temperature	Guinea pig No.	*l*-T.R. mg.	Interval between injections in hours	Result and symptoms after injection of *d*-antigen	Subsequent change in body temperature
				°C.					°C.
67	10	1	Weakness	−4.5	77	10	1	† 4 min.	
68	10	18	Cough, slight weakness	−1.1	78	10	18	† 4 "	
					79	5	1	† 4 "	
					80	5	2½	† 4 "	
69	10	18	Dyspnea spasms	−1.2	81	5	2½	† 5 "	
					82	5	3½	† 4 "	
70	5	1½	Weakness	−0.2	83	5	24	† 5 "	
71	5	2	Negative	+0.7	84	2.5	1	† 5 "	
72	5	2½	Slight	−0.7	85	2.5	2½	Few convulsions, very sick	−3.6
73	5	2½	Negative	−0.3					
74	5	24	Cough	−4.0					
75			Weakness						
75	2.5	1	Somewhat sick, weakness	−1.3					
76	2.5	2	Negative	+0.6					

Animals Sensitized with l-Antigen

Guinea pig No.	*d*-T.R. mg.	Interval between injections in hours	Result and symptoms after injection of *l*-antigen	Guinea pig No.	*l*-T.R. mg.	Interval between injections in hours	Result and symptoms after injection of *l*-antigen	Subsequent change in temperature
								°C.
86	10	16	† 4 min.	96	10	16	Slight, weak	−2.8
87	5	2½	† 4 "	97	10	16	Negative	−1.0
88	5	3	† 4 "	98	5	2½	Negative	−0.2
89	5	3	† 4 "	99	5	3	† 5 min.	
90	5	3	† 5 "	100	5	3	Somewhat weak	−1.0
91	5	3	† 2 "					
92	5	3	† 5 "	101	5	3	Weakness	−1.9
93	5	3	† 4 "	102	5	3	Slight, weak	−0.5
94	5	3½	† 4 "	103	5	3	Weak	−1.8
95	5	4	† 6 "	104	5	3	Negative	−0.4
				105	5	3½	Slight, weak	−1.6
				106	5	3½	Slight, weak	−1.6

acute shock, whilst all animals but one, injected with quantities of 2.5 to 10 mg. of the homologous dye, survived. The surviving animals exhibited but rarely typical anaphylactic symptoms as spasms or cough, although many appeared sick. With higher doses of the dye the protection was still evident even when the injection of the antigen was made the following day.

Another batch of animals was sensitized passively by injecting a potent precipitating immune serum produced in a rabbit by immunization with azoprotein made from horse serum and diazotized *p*-arsanilic acid.[2] On injecting intraperitoneally guinea pigs weighing about 300 gm. with 0.3 cc. of this immune serum, the animals proved to be sensitive to an azoprotein prepared from diazotized *p*-arsanilic acid and chicken serum. The minimal lethal dose was regularly found to be 0.5 mg. In the tests presented in Table VII the animals were passively sensitized with 0.3 cc. of the immune serum. The substance tested for inhibition was a product of coupling diazotized *p*-arsanilic acid and tyrosine (2). This was injected intravenously in a volume of 0.5 cc. at stated intervals prior to the administration of the antigen or in a mixture with antigen (indicated as "0" in Table VII). The antigen was employed in a quantity corresponding to two minimal lethal doses.

From Table VII it is seen that the animals were protected from lethal shock by quantities of 2.5 to 1.25 mg. of the dye regardless of the time interval between the two injections. These animals showed either no symptoms or became somewhat weak. In two animals only were slight convulsions or coughing observed. With 0.6 and 0.3 mg. of the dye the results were irregular but again the outcome appeared not to depend on the time elapsed after the injection. A still smaller dose (0.15 mg.) failed to prevent shock also when the dye was injected simultaneously with the antigen.

A few experiments with solutions of sodium *p*-arsanilate seemed to indicate that also this substance has an inhibitory effect upon the anaphylactic reaction but considerably larger quantities were used than of the azosubstance.

The protection described can be explained in two ways, either by assuming that this effect is similar to the inhibition of the precipitin

[2] For the method see (15).

reaction *in vitro* by simple substances containing the specific group (16), or that the mechanism is analogous to the well known desensitiza-

TABLE VII

Inhibition by the Injection of Azodye

Animals passively sensitized with 0.3 cc. immune serum; the next day injection of the compound of *p*-arsanilic acid and tyrosin, followed by the administration of 1 mg. of the shocking antigen.

Guinea pig No.	Atoxyl-tyrosine in mg.	Interval between injection of dye and antigen in hours	Shocking antigen	Result and symptoms after injection of shocking antigen	Subsequent change in body temperature
					°C.
107	2.5	3	1 mg.	Somewhat weak	−1.6
108	2.5	18	1 "	Few coughs, somewhat weak	−1.6
109	2.5	18	1 "	Negative	+0.55
110	1.25	0	1 "	Vigorous scratching	−1.9
111	1.25	0	1 "	Negative	−1.55
112	1.25	3	1 "	"	−0.75
113	1.25	3	1 "	"	−1.8
114	1.25	18	1 "	Spasms	−2.4
115	0.6	0	1 "	† 4 min.	
116	0.6	0	1 "	Convulsions, weak	−2.3
117	0.6	3	1 "	Spasms	−1.55
118	0.6	3	1 "	Negative	−1.25
119	0.6	3	1 "	"	0
120	0.6	18	1 "	† 4 min.	
121	0.3	0	1 "	Somewhat weak	−1.1
122	0.3	0	1 "	† 4 min.	
123	0.3	3	1 "	Dyspnea, somewhat weak	−1.45
124	0.3	3	1 "	Severe	−8.85
125	0.3	3	1 "	† 6 min.	
126	0.3	4	1 "	Negative	−0.9
127	0.15	0	1 "	† 4 min.	
128	0.15	0	1 "	† 4 "	
129	0.15	3	1 "	Very severe, almost dying	−2.4
130			0.5 "	† 5 min.	
131			0.5 "	† 6 "	
132			0.5 "	† 3 "	
133			0.5 "	† 4 "	
134			0.5 "	† 4 "	
135			0.5 "	† 3 "	
136			0.25 "	Slight to moderate	−3.0

tion by small quantities of antigen. On the first assumption one would expect protection to diminish with the elimination of the inhibiting substance from the blood stream. Actually the elimination takes place rather quickly since soon after the injection the urine is distinctly colored. Further evidence was gained from an examination of the color of the serum of guinea pigs after intravenous injection of the dye and from an estimation of the dye in the serum by inhibition of the precipitin reaction. From the few tests made it appeared that a considerable part of the azodye (about half) was already eliminated within the first hour after the injection.

On the other hand protection was still demonstrable on the day following the administration of the dye, and furthermore, in the experiments of Table VII there was no noticeable difference in the results whether the inhibiting substance was injected simultaneously with the antigen or 3 hours afterwards. Consequently one can conclude that the effect is not due simply to the presence in the circulation of the substances tested but to a desensitizing action upon the tissues. This view is corroborated by the observation that frequently the injection of homologous azodye into sensitized animals was followed by a significant drop in temperature and in a number of cases even by anaphylactic shock (17), but in some series of experiments this result could not be duplicated.

It is possible, however, that there are other instances in which protection is brought about by the same mechanism as inhibition *in vitro*. This is suggested by the results of Tillett, Avery, and Goebel, who noticed that their glucosides prevented shock only when injected just prior to the antigen, but not after an interval of 2 hours. The apparent discrepancy between their results and the present ones can probably be attributed to differences in the chemical nature of the substances used.

SUMMARY

Experiments with azoproteins containing stereo-chemical isomeric groups of *d*- and *l*-tartaric acid showed well marked specificity of the anaphylactic reaction to these antigens, in conformity with the results of precipitin tests.

Shock in these animals could be prevented by injection of azodye containing the specific groups. This phenomenon is ascribed to a desensitization.

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SURVIVAL AND MULTIPLICATION OF THE VIRUS OF POLIOMYELITIS IN VITRO

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The subject of the artificial cultivation of filterable viruses has again brought into prominence the experiments of Flexner and Noguchi (1) and their followers with the so-called globoid bodies of poliomyelitis. The present paper deals with an attempt to repeat the results of the studies of Flexner and Noguchi and it is therefore desirable to present in brief form the results of the earlier work.

The original paper of Flexner and Noguchi (1) described the globoid bodies as minute, formed structures, cultivated by specially devised methods from tissues of the central nervous system from both human and experimental sources. The tissues were either fresh or glycerolated, unfiltered or filtered through Berkefeld V or N candles.

Methods of Cultivation.—The culture medium consisted essentially of human ascitic fluid to which had been added a fragment of sterile fresh tissue, usually rabbit's kidney. The liquid was covered with a deep layer of sterile paraffin oil and of the two sets of cultures, one was placed in an anaerobic jar and the other kept outside. All tubes were incubated at 37°C.

In the tubes kept outside the jar, it was observed after 5 days that about the fragments of tissue an opalescence appeared which could be gradually diffused through the tube by gentle shaking. After another period of 3 to 5 days, the opalescence extended into the upper portion of the medium, but at the expiration of 10 to 12 days the diffuse but slight opacity began to diminish, and irregular particles formed and slowly fell to the bottom of the tube. In control tubes, either no change was observed, or a slight granular precipitate covered the tissue; and, while in tubes in which growth had been noted the opalescence increased in extent, in the control series the precipitate gathered progressively about the tissue fragments. In the corresponding tubes kept in an anaerobic jar, a similar opalescent growth was seen, although it was slighter than that just described and required a somewhat longer time to reach its full development.

For the demonstration of colony formation, a semisolid medium was used which comprised ascitic fluid and sterile rabbit kidney, to which sufficient 2 per cent

agar had been added to produce a solid mixture. This medium was not successful for initial growths, but after about the third generation of growth in fluid medium, transfer could be effected to the solid medium. In this, growth was noted after several days as a diffuse opalescence which first appeared about the tissue fragments and gradually aggregated into visible, minute colonies. Later, the opalescence rose in the medium until it reached about 3 cm. below the surface, the upper limit of growth being sharply demarcated.

In the original paper, mention was made of the fact that initial growth could be secured in a medium from which either rabbit kidney or ascitic fluid had been eliminated, the former being replaced by using a somewhat larger fragment of poliomyelitic brain or the latter by adding brain tissue extract or sheep serum water. Cultures could not be obtained in the absence of both kidney tissue and ascitic fluid, which might each have contained living cells; modified media were inferior generally to a mixture of the above two components.

The results obtained by the use of the substituted media indicate that growth was derived primarily from the inoculum and not from the kidney tissue or ascitic fluid. The original observation supporting this view is that relatively greater success was obtained when fragments rather than emulsions of poliomyelitic tissue were used. For it has been found that bacteria may be introduced from the outside into cultures of brain tissue from herpes-virus encephalitis (2) and from poliomyelitis (3) during the process of grinding the tissue in the preparation of emulsions.

Characteristics of the Globoid Body.—The globoid bodies were originally described as minute, globular structures, measuring from 0.15 to 0.3 micron in diameter, and arranged in pairs, chains, and small masses, according to the nature of the medium. In fluid medium, pairs and chains predominated; within the tissues of poliomyelitic patients or infected monkeys, only pairs or small masses were discernible, never chains. The reaction to Gram's stain was variable, that is, the bodies retained the stain feebly in earlier generations, but more intensely in older cultures. In peptone medium the organisms appeared to be larger and retained the Gram stain more firmly.

In initial cultures, considerable difficulty was encountered in demonstrating the bodies during the first 4 or 5 days. They could be detected more readily on the sixth or seventh day, and maximum growth was obtained from about the eighth to the twelfth day. After 3 or 4 weeks, however, the bodies became enlarged and irregularly stained, although very minute structures, barely visible, were still present. These altered cultures were filterable through Berkefeld filters, just as were the globoid organisms.

It would appear, therefore, from the original description in which the authors stressed the ability of the globoid bodies to form definite colonies and to show a distinctive morphology, that the bodies were unequivocally organisms.

Pathogenicity in Monkeys.—The original paper emphasized the point that only exceptional cultures possessed pathogenicity in the monkey. Furthermore,

the limit beyond which the virus itself was considered not to be infective was that represented by a second transfer of the original virus in the culture medium.

Two sets of inoculation experiments were reported: one comprising fluid and solid cultures of globoid bodies derived from four human cases, and the other, cultures from the M.A. virus highly adapted by prolonged propagation in monkeys.

The mixed cultures of the human strains, in the third generation, were inoculated into a monkey, which developed characteristic experimental poliomyelitis. A second passage from this animal to two normal monkeys, and later, a third passage to a single *rhesus*, proved successful. From three of these four animals, cultures of the microorganisms were recovered.

The monkey strains of globoid bodies were successfully inoculated as follows: One monkey was injected with a mixed culture representing the third and fifth generations, and a second, third and fourth animal, with fifth to sixth generation cultures, all developed typical poliomyelitis. The globoid bodies were recovered in cultures made from the brains of all these animals. Furthermore, from the second and third *Macacus rhesus* successful passage was effected through two successive series of animals. In addition, a fifth monkey was inoculated with a mixed culture representing the eighteenth and twentieth generations. This animal died on the third day from a secondary infection but the inoculation of a filtrate of the site of inoculation in the brain into a normal *cynomolgus* was followed, after 13 days, by characteristic experimental poliomyelitis.

In one instance in the foregoing experiments, mention was made of the fact that characteristic experimental poliomyelitis was induced with a culture derived from an affected monkey which, in turn, had also been inoculated successfully with globoid bodies.

To sum up, the original report offered evidence of specific pathogenicity of globoid bodies even to the eighteenth or twentieth generation and of the successful recovery of a virulent culture from an animal injected with the globoid bodies themselves.

In a later article, Flexner, Noguchi, and Amoss (4) extended the studies on the globoid bodies and found that the microorganisms survived in the semisolid medium kept at room temperature or at 37°C. for over 13 months. A test for virulence showed these cultures to be pathogenic 6 months later, or 19 months after isolation. In this communication the authors also reported the preparation of "mass" culture medium and the employment of the prodigious number of globoid bodies so obtained for pathogenicity tests. The mass cultures were injected three or four times, since the earlier studies had revealed that only exceptional growths were active and that repeated injections of the ordinary virus (5) sometimes induced paralysis. Thus, one *rhesus* monkey was inoculated intraspinally four times at intervals of 6 to 11 days with mass cultures representing the fourth to thirteenth generations. 5 days after the last injection the monkey developed characteristic experimental poliomyelitis. Another monkey was injected intraperitoneally four times with the same material, and at the same intervals as in the preceding

experiment, and typical poliomyelitis was induced. The nervous tissue of these two monkeys, glycerolated for 6 and 70 days respectively, proved infectious. The effect of the repeated injections is cumulative, for it was shown by a control test that the final, or fourth injection, by itself, failed to affect a normal animal.

The experiments described led to the inference that there is a wide fluctuation in pathogenic action of the globoid bodies, and only exceptional strains are infective. In addition, it was emphasized that the recovery of the bodies in culture from affected monkeys is made only with difficulty. Finally, it was pointed out that, by means of mass cultures, the bacterial nature of the globoid bodies is unequivocally established.

The next communication, by Amoss (6), again presented evidence to indicate the fact that the globoid bodies are microorganisms, and that after long cultivation *in vitro* they become saprophytic and grow more readily in a considerable variety of media. Moreover, Amoss reported the recovery of two additional, but nonpathogenic, cultures from monkeys with experimental poliomyelitis induced by human and M.A. virus. He emphasized in this paper a fact that had already been pointed out in the previous articles (1, 4), namely, that highly parasitic cultures are refractory to artificial cultivation.

Smillie (7) contributed a series of twenty-two cultures of globoid bodies in fluid and semisolid media obtained from the tissues of seven monkeys with experimental poliomyelitis. Of these, eight different strains were inoculated into corresponding *rhesus* monkeys. Five animals were unaffected but three, receiving fourth and fifth generation growths, showed some degree of paralysis after intracranial or intraspinal injection. These latter reactions could not, however, be definitely proved as poliomyelitic. Smillie also found that established cultures can survive in the icebox for months; that dye indicators inhibit their growth; that the preferable pH of the medium is at blood neutrality; that strict anaerobiosis is essential and that body fluids, especially ascitic fluid, are required for their proper development. Most noteworthy is the fact, as shown also by Amoss (6), that after having once become established and accustomed to artificial media, the globoid bodies grow more readily and may be more easily transferred.

The observers mentioned in the preceding paragraphs have therefore defined the characteristics of a true culture of globoid bodies. In view of the minute size of the microorganisms, emphasis was laid on the possible confusion which could arise in distinguishing them from granular precipitates in the culture medium. Hence the criteria of a culture should comprise the following principles: (a) characteristic growth in special, anaerobic, fluid medium; (b) definite colony formation; (c) distinctive growth in mass cultures, and (d) agreement with the other properties already mentioned in the foregoing résumé. Finally, (e) in respect to pathogenicity, attention has been called by

the early investigators to the fact that only exceptional cultures show infectiousness and that the saprophytic strains are cultivable with difficulty while parasitic ones are even more refractory. On the other hand, definite experimental poliomyelitis has been induced in monkeys with the first, third, fourth, fifth, sixth, eighth, eighteenth, and twentieth generations of the globoid bodies. The eighteenth generation has been calculated to represent a dilution of $1:24^{17}$ of the original virus—considerably beyond the limits of its inherent infectivity. In one instance, a culture in semisolid medium, in its eighth generation and kept at 37°C. for 13 months, had not lost its infectiousness, whereas ordinary virus in a similar medium survives no longer than 20 to 30 days (4). Furthermore, the microorganism has been recultivated from the tissues of monkeys in which poliomyelitis was induced by the globoid bodies (in one series, from all of four cases) and such strains have, in turn, again produced the experimental disease.

With this definition of a culture of globoid bodies in mind, we can now compare the results of other investigators with those of Flexner and Noguchi (1, 4), of Amoss (6), and of Smillie (7).

In 1918, Heist, Solis-Cohen, and Kolmer (8) reported the isolation from human and monkey poliomyelitic material of four different strains of globoid bodies. These cultures agreed morphologically and culturally, in fluid, semisolid, and mass-culture medium, with the descriptions originally given (1, 6, 7). Furthermore, a definite distinction was made by Heist and Solis-Cohen (9, 10) between globoid bodies and ordinary streptococci—a finding also reported by Smillie (7) and by the prior investigators. Although the cultures of Heist and his co-workers were carried successfully through at least ten generations, no inoculation tests were made.

On the other hand, the results of the cultivation tests reported by Tsen (11) are not so clearcut. After "many trials" he succeeded in finding organisms which he believed to be similar to globoid bodies. But they could not be maintained for more than three generations, and hence were not inoculated into animals. Since only fluid medium was used, and the "organisms" therein disappeared rapidly, it is likely that Tsen may have confused granular precipitate with the globoid microorganisms.

Into this category of granulations may also be placed the so-called globoid body cultures obtained by Foster (12) from the nasopharyngeal secretions of common cold cases, and those of Bradford, Bashford, and Wilson (13). Olitsky and McCartney (14), in discussing the significance of Foster's cultures, pointed out how the error of confusing precipitates with minute organisms may arise and, further-

more, attempted to indicate the methods by which such misinterpretations may be eliminated. In the case of Bradford and his co-workers, their cultures of so-called "globoid bodies," obtained from patients with influenza, trench fever, war nephritis, and a number of other diseases, were later shown to consist of artefacts (Arkwright—15)—a view which the investigators themselves finally accepted.

In a study concerning the etiology of epidemic encephalitis lethargica, Loewe and Strauss (16), using the fluid and semisolid culture media already described (1), recovered what was believed to be a minute, globular organism from the brain, from nasopharyngeal mucous membrane tissue and washings, from spinal fluid, and from blood of cases of the affection. These investigators stated that their filterable organism resembled in morphology and cultural characteristics the globoid bodies, but that it possessed distinctly different pathogenic effects. They have not, however, reported the distinctive bacteria-like growth, as described by Amoss (6), in mass-culture medium. The fact, however, that colonies were obtained indicates the occurrence of bodies of microorganismal nature in their cultures, which, the authors believe, should be placed in the globoid body group.

EXPERIMENTAL

As indicated in the introductory paragraph, we undertook to repeat the earlier work on the cultivation of the globoid bodies and, especially, to secure cultures which possessed pathogenic properties.

For this purpose, we had access to abundant material taken from monkeys which had developed experimental poliomyelitis after the inoculation[1] of a highly active mixture of two virus strains—M.A. and K—(17, 18) which had been propagated for many years in monkeys. The animals, as soon as definitely paralyzed and before becoming moribund, were etherized and the brain and spinal cord were removed with sterile precautions. The tissues were generally free from secondary bacterial contamination, or bacteria derived, during exposure, from the air. Inoculation of media was immediately carried out, using fragments of brain or spinal cord, or a Berkefeld V filtrate of a 5 per cent physiological saline suspension of these tissues.

Cultivation

The culture medium was that of Noguchi, consisting of ascitic fluid and a fragment of sterile, fresh, rabbit kidney.

In the preparation of initial cultures, we attempted to repeat precisely the original method described by Flexner and Noguchi (1). The fragment of rabbit kidney

[1] All inoculations in the monkey were carried out under ether anesthesia.

was placed in a test tube measuring 1.5 x 20 cm. The inoculum, consisting of either a small fragment of brain or of spinal cord, or of 0.5 cc. of the filtrate of these tissues, was next added and then about 15 cc. of sterile ascitic fluid. Finally, about 2 cc. of sterile liquid albolene was added in such a way as to form a layer over the surface of the medium. One set of tubes of each series was placed in the Boëz anaerobic jar (19), and a duplicate set kept outside. The incubation was at 37°C.

A modified technique was also used: sufficient 1 per cent neutral cysteine hydrochloride solution was introduced into the ascitic fluid to give a final concentration of 1:2000. The cysteine medium was sealed with solid petrolatum instead of liquid albolene. We found, however, that this modification did not lead to an increase in the number of positive "cultures."

As a rule, about 50 tubes of medium were inoculated during each original attempt at cultivation: two-thirds of them were set up as first described, and one-third with the cysteine.

After 10 to 14 days, the tubes were removed from the thermostat and a small amount of the medium was withdrawn from the bottom of each tube with a sterile capillary pipette, for film preparations. These preparations were allowed to dry in the air and, after being fixed by heat, were stained for 3 minutes with well-ripened, alkaline, methylene blue.

All tubes found by microscopic examination to be contaminated with ordinary, familiar bacteria were discarded. On the other hand, each culture thought to contain material resembling globoid bodies was subplanted to six or eight tubes of Noguchi's fluid medium and upon two rabbit-blood, dextrose-agar plates. In the case of each subplant, one-half the number of tubes and plates was placed in an anaerobic jar and the other half kept outside. In addition numerous uninoculated control tubes of the medium, and inoculated control tubes of dextrose broth were included in each series of transfers.

No characteristic macroscopic changes were visible in the inoculated tubes at any time. The opalescence described by Flexner and Noguchi (1) as appearing around the kidney at the end of the fifth day was not detected but a distinct clouding, possibly due to autolysis, was noted at the end of 24 hours.

With regard to the formation of colonies, which other workers (Flexner and Noguchi—1; Flexner, Noguchi, and Amoss—4; Amoss—6; Smillie—7, and Heist, Solis-Cohen, and Kolmer—8) agree in emphasizing as characteristic of the globoid bodies, and as indicative of their microorganismal nature, we were unable to secure any evidence. This may perhaps have been due to the fact that we did not employ the semisolid medium of Noguchi. However, no colonies could be detected on the plates, incubated anaerobically, which were made from tubes showing suspected growth. Nor were we successful in obtaining growth in the special mass-culture medium (4), although no less than twelve attempts were made by this method.

In view of our having used different media in this part of the work, any comparison to be made between our results and those of the original investigators should be based on findings in fluid cultures.

To recapitulate: Efforts at cultivations were made with fragments of the brain or cord or with Berkefeld V filtrates of the nervous tissues of seven monkeys inoculated with poliomyelitic virus and suffering from experimental poliomyelitis. With these materials, 315 tubes were inoculated, thirty-six of which showed minute morphological particles or "bodies" suggesting microorganisms. The subplanting from these "positive" tubes was disappointing, owing to the failure to obtain secondary growths in later subplants or to failure due to contamination with ordinary bacteria. Thus, while all seven monkeys yielded minute particles suggesting growths in initial cultures, the "growths" derived from three animals failed after the first subplant, from one after the third, from one after the fourth, from one after the eighth, and from the last monkey after the eleventh subplant.

Morphology

The microscopic appearances to be described relate wholly to the fluid subplants and it should be recognized that because of the presence of the autolyzing kidney fragment and the resulting granular precipitate, it is just such cultures that are most subject to misinterpretations. Indeed, the distinction in early subplants between minute microorganisms and precipitate is so indefinite that we were led to apply the method of cataphoresis in order to attempt possible differentiation. We already knew that the poliomyelitic virus in nervous tissue suspensions migrates to the anode (20). Applying the same experimental procedures (21) to washed "cultures" in twenty-three tubes derived from six different "strains" in five separate tests, no conclusive evidence could be obtained, by this method, of differentiation of "microorganisms" from precipitate. For whatever picture was detected at the anode could be seen also at the cathode. On the other hand, control experiments with *Bacterium pneumosintes* and *Bacterium coli* were clearcut; there was active migration of both to the anode.*

Notwithstanding these facts, we were able to pick out thirty-six from 315 tubes in which the distinctions were sufficiently wide to suggest the possibility of microorganisms being present. The main points of

* In carrying out the cataphoresis experiments, we were aided by the effective cooperation of Dr. D. C. Hoffman of The Rockefeller Institute.

such distinctions are as follows: In early subplants, the minute and "globoid bodies" were single, in pairs, or in short chains, the individual bodies being of a fairly uniform appearance. In older subplants, the bodies were more numerous and in part formed agglomerated masses. In remote subplants, they were somewhat larger. The bodies stained well with well-ripened alkaline methylene blue and, as a rule, did not retain Gram's stain. In stained specimens the "bodies" appeared to be raised above the background of amorphous material, cellular detritus, and indefinite particles and were not refractile. It should be mentioned that in practically all film preparations we observed well-preserved kidney tissue cells and leucocytes, the latter probably derived from the ascitic fluid.

Power to Infect Monkeys

We pass now to the most significant part of our study, namely, the power of the "cultures" to infect and induce experimental poliomyelitis in monkeys. Indeed, in one series of subplants, derived from the brain and cord of a monkey experimentally infected with the virus described in Experiment 1, we were successful in producing experimental poliomyelitis in monkeys with the materials taken from "cultures" in the seventh, eighth, ninth, and tenth transfers. It should be noted that the seventh transfer represents a dilution of the original material cultivated of about 1.5×10^{-12}, and the tenth, of about 1.3×10^{-18}. The history of the monkey yielding the active material is as follows:

Experiment 1.—Macacus rhesus inoculated intracerebrally on February 8, 1929, with 1 cc. of the suspension of anodic material obtained by cataphoresis of brain and cord tissues from a monkey in the paralytic stage of the experimental disease (20). February 19, excitement, tremor, right facial paralysis, and ptosis were noted. February 20, ataxia, tremors, weakness of right arm and shoulder and both legs were observed. February 21, the animal was prostrate and unable to move its arms and legs. Etherized. The gross and histopathological examinations revealed characteristic lesions of experimental poliomyelitis.

Sixty-nine tubes were inoculated with the infected nervous tissue of this animal.

Of the sixty-nine tubes, eight showed what appeared to be a minute, "globoid microorganism." These were subplanted to sixteen tubes of which, after 12 days' incubation, five were "positive." A third subplant into thirty tubes yielded sixteen; a fourth into forty-two tubes yielded nine; a fifth of forty-five, eleven; a sixth of sixty-six, twenty; a seventh of sixty, thirty; an eighth of forty-eight,

thirty-seven; a ninth of thirty-two, four; and a tenth of thirty-two, five "positives." All the forty tubes of the eleventh subplant were contaminated by moulds and diphtheroid organisms. By the time the sixth subplant was reached, all the "positive" tubes had a common origin from one tube of the first transfer.

The medium is notably irregular and uncertain in its composition because of the variations in quality of the ascitic fluid and possibly of the kidney tissue. Noguchi (22) originally pointed out that certain samples of ascitic fluid were more suitable than others for cultivating spirochetes and other investigators have emphasized this factor as influencing cultures of globoid bodies (4, 6, 7). That uncontrolled fluctuating factors played a part in our results is indicated by the discrepancies arising in the several subplants. The irregularity in positive tubes is correlated with the number of "bodies" visible under the microscope: In the first three subplants they were few, from the fourth to the eighth subplant, many; with the seventh subplant they predominated, but in the ninth to eleventh they became very few and difficult to find.

Monkeys were inoculated with materials from the seventh, eighth, ninth, and tenth subplants of these "cultures." The material inoculated consisted of centrifuged sediment of the tubes, washed, as a rule, five times with physiological salt solution. In one instance (Experiment 2) to the washed sediment of the seventh subplant was added the unwashed sediment of the sixth and fourth subplants.

Experiment 2.—Macacus rhesus A. April 18, 1929, pooled material from the bottom of the positive tube in the sixth transfer of the culture obtained in Experiment 1 (hereafter designated as Bodies 1) was prepared and of this mixture 2 cc. were injected intracerebrally, 10 cc. were injected into the peritoneal cavity and 2 cc. into the spinal canal.

April 23, pooled material from the bottom of the positive tube in the fourth transfer of Bodies 1 was prepared, and of this mixture 2 cc. were injected intracerebrally, 9 cc. intraperitoneally, and 2 cc. intraspinally.

May 1, the material from the bottom of the positive tube of the seventh transfer was centrifuged at high speed for 15 minutes; the sediment was suspended in 3 cc. of physiological saline solution, and then centrifuged again. This was repeated three times. 1.75 cc. of the final suspension were injected into the left cerebral hemisphere.

May 11, tremor; right facial paralysis; ataxia; complete paralysis of left arm, and legs very weak.

May 12, prostrate. Etherized.

Autopsy.—Meninges normal. A very small area of softening was visible in the border of the right internal capsule, involving the lenticular nucleus. This site contained old blood and yellow pigment. The midbrain showed spotty translucent areas about 1 to 2 mm. in diameter. The cervical cord contained pink, translucent, soft areas in the region of the anterior horn cells.

Microscopical Examination.—Forebrain: A moderate mononuclear cellular reaction was present. Lateral to and partly involving the lenticular nucleus was a small area of softening containing red blood cells in various stages of degeneration. Around this area were many phagocytic microglia. Certain of the nerve cells showed varying degrees of degeneration and some of them were phagocytized.

Medulla: A slight mononuclear infiltration of the pia-arachnoid membrane was seen. Moderate perivascular infiltration was present. A certain amount of nerve cell degeneration with and without accompanying cellular reaction occurred.

Cord: A slight lymphocytic reaction was observed in the pia-arachnoid membrane, together with a marked perivascular mononuclear infiltration. In the cervical region there was almost complete destruction of both the anterior and posterior horn cells, with accompanying phagocytosis of the débris by microglia. The same reaction was present but was progressively less marked in the lower levels of the cord.

A 5 per cent saline Berkefeld N filtrate was prepared from portions of the cord and of this 1 cc. was injected intracerebrally into a monkey. This monkey developed typical experimental poliomyelitis within 6 days.

Cultivation experiments in Smith-Noguchi medium were carried out with fresh central nervous tissue from Monkey A. Seventy-one tubes were inoculated and after 14 days' incubation, five tubes contained bodies resembling those with which Monkey A had been inoculated.

Experiment 3.—Macacus rhesus B. May 1, 1929, 2.5 cc. of a saline suspension of washed materials from the bottom of the positive tube of the seventh transfer of Bodies 1 were injected into the left cerebral hemisphere.

May 6, slight tremor; ataxia; marked left facial paralysis; both arms practically completely paralyzed.

May 7, prostrate; facial paralysis increased. Etherized.

Autopsy.—At a site of inoculation there was a soft area 2 x 2 mm. containing a small amount of golden yellow pigment. No cyst formation or frank hemorrhage was observed. The region of the anterior horn cells in the spinal cord showed injection and slight hemorrhage. This was most marked in the cervical region.

Microscopical Examination.—Brain: Slight perivascular lymphocytic reaction in the region of the thalamus. Irregular nerve cell degeneration. The choroid

plexus was infiltrated with lymphocytes. In the sub-arachnoid space was a diffuse lymphocytic and mononuclear cellular reaction.

Medulla: Foci of mononuclear cells, some surrounding degenerated nerve cells, were present in the region of the fourth ventricle. A marked perivascular mononuclear infiltration occurred throughout the medulla. All stages of nerve cell degeneration were seen.

Cervical cord: A moderate meningeal and perivascular reaction was observed. The neurons showed marked destruction and active neuronophagia.

Thoracic cord: The meninges were thickly infiltrated with lymphocytes. The cells of the anterior and posterior horns showed marked acidophilic degeneration, but their outline was preserved. The nerve cells were surrounded by a large number of polymorphonuclear and mononuclear leucocytes. The same changes were observed in the lumbar cord.

Intervertebral ganglia: There was a marked interstitial lymphocytic reaction and a moderate acidophilic degeneration of the nerve cells.

Five monkeys were inoculated with suspensions and filtrates prepared from the cord of Monkey B. Three of these control monkeys developed typical experimental poliomyelitis within 7 days; the remaining two were unaffected.

Cultivation experiments in Smith-Noguchi medium were made with fresh material from the brain and spinal cord of Animal B. In the second transfer bodies closely resembling those inoculated were isolated.

Experiment 4.—Macacus rhesus C. May 15, 1929, 1.5 cc. of a saline suspension of washed material from the bottom of the positive tubes of the eighth transfer of Bodies 1 were inoculated into the left cerebral hemisphere and 1.5 cc. of the same suspension were injected into the spinal canal.

May 21, tremors; slight ptosis of both upper eyelids; ataxia; partial paralysis of both arms and shoulders; legs very weak. Etherized.

Autopsy.—Meninges normal. Site of inoculation used for touch preparations. A definite small hemorrhage was seen in the striate body. The cervical cord revealed definite areas of congestion and hemorrhage in the region of the anterior horn cells. These changes were less marked in the lumbar and sacral cord.

Microscopical Examination.—Site of inoculation: No bodies were seen in touch preparations. Lateral to the anterior part of the striate body was a narrow zone of softening containing red blood cells, granular débris, and a moderate amount of pigment. About the periphery of this area was a large number of glial nuclei.

Cord: The anterior horn cells showed varying degrees of degeneration, some being slightly involved while others were completely destroyed. A diffuse cellular infiltration of polymorphonuclear and mononuclear leucocytes was present. This reaction became less marked in the lumbar and sacral regions.

Intervertebral ganglia: A diffuse interstitial lymphocytic infiltration was present and a moderate number of nerve cells showed partial or complete degeneration.

A 5 per cent saline Berkefeld N filtrate was prepared from the brain and cord of Monkey C. 1 cc. of this filtrate was injected into the left cerebral hemisphere of a normal control monkey. Within 8 days the animal developed typical experimental poliomyelitis.

Cultivation experiments in Smith-Noguchi medium were made with fresh tissue of the central nervous system from Monkey C. After 14 days' incubation, one of forty-seven inoculated tubes contained bodies similar to those with which the monkey had been inoculated.

Experiment 5.—*Macacus rhesus* D. May 31, 1929, 1.25 cc. of a washed saline suspension of the material from the bottom of the positive tubes of the ninth transfer of Bodies 1 were injected into the right and left cerebral hemispheres.

June 6, tremor; ataxia; both arms paralyzed; legs weak.

June 7, prostrate. Etherized.

Autopsy.—The meninges were slightly injected. The left site of inoculation showed an old hemorrhage with some yellow pigment and an area of softening extending into the internal capsule and the caudate nucleus. A similar picture was present in the right site of inoculation. Typical translucent, congested and hemorrhagic areas were visible in the medulla and in the region of the anterior horn in the cervical cord. No organisms were seen in preparations made from the sites of inoculation.

Microscopical Examination.—The site of inoculation showed a hemorrhage with hemosiderin pigment and was surrounded by phagocytic mononuclear cells. A slight diffuse increase in glial nuclei was present. Nearly all of the anterior horn cells in the cervical region had disappeared. Many foci of phagocytic mononuclear cells were present. A moderate perivascular lymphocytic reaction was observed.

A 5 per cent saline suspension was made of the cord of Monkey D. 0.5 cc. was inoculated into the left cerebral hemisphere of a normal, control monkey, which in the course of 7 days developed typical experimental poliomyelitis.

Cultivation experiments in Smith-Noguchi medium were made with the fresh central nervous tissue from Monkey D. None of the forty-eight tubes showed bodies similar to those with which Monkey D had been inoculated.

Experiment 6.—Macacus rhesus E. May 31, 1929, 1.25 cc. of a washed saline suspension of the material from four negative tubes of the ninth transfer of Bodies

1 were inoculated into the right and left cerebral hemispheres. This material contained no demonstrable bodies.

The monkey remained well and was discarded after 1 month's observation.

Experiment 7.—Macacus rhesus F. June 14, 1929, 1.5 cc. of washed saline suspension of material from the bottom of five positive tubes of the tenth transfer of Bodies 1 were injected into the right and left cerebral hemispheres.

June 20, excited; tremors; right deltoid weak.

June 21, prostrate. Etherized.

Autopsy.—There was slight meningeal congestion. An area on the left side, greenish gray to red in color, was filled with tenacious, soft, elastic material which contained staphylococci. The area extended from the superior surface of the frontal lobe to a region near the internal capsule, 1 to 2 mm. wide, and spread out at the base to a pyramidal area about 4 mm. in diameter. On the right side a similar condition prevailed, although the area was not as large but more hemorrhagic and extended almost to the internal capsule. The fourth ventricle revealed the usual picture of poliomyelitis, but its edges were bulging and the glistening surface showed pink, turgid, tiny, translucent, somewhat hemorrhagic areas. The cervical cord exhibited the characteristic injection and translucence of the anterior horns, but in the dorsal and lumbar cord the pinkish tinge was not as prominent.

Microscopical Examination.—Forebrain: At the site of inoculation an elongated area was seen extending from near the cortex to the external surface of the lenticular nucleus. This area contained erythrocytes, polymorphonuclear and endothelial cells, and granular débris. A small amount of golden yellow pigment was present. The tissue forming the border of this area comprised a mass of phagocytic mononuclear cells and proliferated neuroglia; there was also perivascular infiltration by lymphocytes. Lymphocytes infiltrated the pia-arachnoid. In the medulla there were present occasional nerve cell degeneration and neuronophagia with a moderate perivascular infiltration.

Cord: Cervical region. Moderate lymphocytic infiltration of the meninges. Almost all neurons showed acidophilic degeneration and some were in the process of neuronophagia. The gray matter was diffusely infiltrated with lymphocytes and with occasional polymorphonuclear cells.

Thoracic region: Here the cord was the seat of an intense reaction. No normal appearing nerve cells were present, all being degenerated and fragmented, the subject of active neuronophagia. Very marked extra-adventitial infiltration was noted.

Lumbar region: Lesions of meningeal mononuclear infiltration; nerve cell degeneration; neuronophagia; perivascular infiltration with some diffuse lymphocytic and mononuclear cellular infiltration throughout the gray matter. In general, the reaction was less marked here than in the cervical or thoracic cord.

Intervertebral ganglia: Moderate diffuse lymphocytic and mononuclear cell infiltration; a few necrotic nerve cells in various stages of degeneration with some showing neuronophagia.

A 5 per cent saline suspension was made of the cord of Monkey F; 0.5 cc. was inoculated into the left cerebral hemisphere of a normal monkey, which developed typical experimental poliomyelitis after an incubation period of 7 days.

Cultivation experiments in Smith-Noguchi medium were made with fresh tissue of the central nervous sytem of Monkey F. Two of twenty-eight tubes showed bodies similar to those employed in the inoculation of Monkey F.

Experimental 8.—Macacus rhesus G. June 14, 1929, 1.5 cc. of a washed saline suspension of the material from five negative tubes, that is, free from "globoid bodies," of the tenth subplant of Bodies 1 were inoculated into the right and left cerebral hemispheres. The monkey remained well throughout the period of observation.

One observes from the preceding experiments that the inoculation in monkeys of a saline suspension of the washed sediment from Smith-Noguchi cultures of poliomyelitic tissue induced, in the seventh, eighth, ninth, and tenth transfers, the clinical syndrome and the pathological effects characteristic of experimental poliomyelitis. It is noteworthy that in the successful experiments, the washed material contained small bodies resembling the globoid bodies described by Flexner and Noguchi. Equally important is the fact that in parallel experiments with material derived from the ninth and tenth transfers, the washed sediment of tubes showing similar bodies was active in monkeys while that of tubes not containing the bodies was inactive.

SUMMARY AND DISCUSSION

The study here reported concerns attempts at bacteriological cultivations with fragments of brain or cord, or with Berkefeld V filtrates of the nervous tissues, from seven monkeys successfully inoculated with poliomyelitic virus. With these materials, 315 tubes were inoculated, of which thirty-six showed minute bodies resembling the globoid bodies described by Flexner and Noguchi. However, a study of subplants from these minute, morphological particles did not convince us that we had in hand actual cultures of the globoid bodies, or indeed of any living microorganism.

Nevertheless, when washed sediments from subplants of one of the

strains, representing the seventh, eighth, ninth, and tenth transfers, were inoculated into monkeys, the clinical signs and pathological effects characteristic of experimental poliomyelitis could be induced. The virulence of the "cultures" could not be ascribed to carrying over the original material into these remote subplants since the seventh transfer represented a dilution of the original cultivated material to about 1.5×10^{-12}, and the tenth, to about 1.3×10^{-18} if one assume, as the transfer technic justifies, a thorough mixing of the contents of each tube. On the contrary, it appears as if the poliomyelitic virus had multiplied *in vitro*, and had increased as a consequence of being in a medium of a modified living tissue-cell culture. For in practically all specimens we observed many well-preserved kidney tissue cells and leucocytes, the latter probably derived from human ascitic fluid, a component of the Smith-Noguchi medium. In this connection, it should be mentioned that the several lots of ascitic fluid used in the cultivation tests were recently obtained from patients and employed from a week to a month after their collection.

There remains for consideration the problem of the selective pathogenicity of the "cultures:" only the material of those tubes of the ninth and tenth transfers which showed the "globoid bodies" proved pathogenic; those respective tubes of the same transfers which were free from the minute bodies but apparently identical in all other respects, were avirulent. It may be that the virus was adsorbed to the particular bodies which we have found in the "cultures" and which resemble closely the globoid bodies of Flexner and Noguchi. Further elaboration of this study would be necessary, however, before such an inference could be regarded as a definite hypothesis.

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A METHOD FOR SUPRAVITAL STAINING OF ANIMALS WITH NEUTRAL RED AND ITS PRESERVATION IN PARAFFIN SECTIONS

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The only method which satisfactorily preserves neutral red in sections of tissue supravitally stained is that reported by Cash (1)—or certain modifications of it—for the study of monocytes and epithelioid cells in the lungs of animals experimentally infected with tuberculosis.

Cash slowly injected into the ear vein 15 cc. of a 1 per cent solution of neutral red, dissolved in normal salt solution. In this manner he secured a true supravital staining of the connective tissue cells of the lungs. Any damage to the cells brings about diffuse staining of cytoplasm and nuclei and obscures those structures which are significant in the so-called supravital reaction. It is an essential part of the technique, therefore, to secure this reaction first. 10 minutes after the injection the rabbit was quickly killed. Thin slices of the lungs were fixed in Zenker-formol solution for from 12 to 24 hours. These blocks of tissue were quickly dehydrated, without washing, in acetone, cleared in benzene or xylol, and rapidly embedded in paraffin. Thin sections were then stained with various dyes, avoiding alcohols and water as much as possible. This method gave uniformly good results with lung tissue.

Prior to the experiments of Cash, McJunkin (2) reported a method for staining tissue supravitally and embedding it in paraffin. He exsanguinated animals from the carotids under light ether anesthesia and then injected saline solution, saturated with neutral red, directly into the tissues. The tissues were fixed in a Zenker formol solution. McJunkin states that the contact with the dye is irregular and that no observations can be made within the areas in which the cells are injured. Indeed, the weakness of the interstitial method of introducing the neutral red is the unevenness and uncertainty of obtaining a supravital reaction of the cells.

Gardner (3) states that the technique of McJunkin proved to be inapplicable

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for use on lung tissue and he therefore modified the methods of McJunkin and Cash. He injected the dye solution either intratracheally or intravenously. The fixation was carried out in the same fluid as that used by McJunkin, except that the formaldehyde was carefully adjusted to a pH of 7.6 with sodium hydroxide. Gardner claims that the sodium has a specific mordanting effect upon staining.

It is well-known that the supravital technique of staining monocytes in the blood or tissues is the most reliable method we have for the accurate differentiation of these cells from other cytological elements. Good descriptions of it can be found in the papers of Simpson (4) and Sabin (5). Forkner (6) has recently described in some detail the methods, as used in Dr. Sabin's laboratory, of applying the technique to tissue cells. This description includes some minor modifications in the methods as originally reported by the above authors. Forkner (7) has shown that lymph nodes from certain areas of the body contain an abundance of monocytes, but that the exact relations of these cells in the tissues could not, by the methods then available, be demonstrated. The technique here recorded was elaborated for the study of this point. It has been found to be equally applicable for the study of all supravitally stainable cells throughout the body. The process of staining and rapid fixation was developed by the writer. The method of dehydration and embedding is a modification of that recorded by Cash (1), to whom I am indebted for suggestions in the development of this technique.

Method

Apparatus.—An instrument which has been of great value in this work is a modified, self-retaining cannula. It is made by inserting a fish-hook (Fig. 1, *a*), the curve of which has been eliminated by heating and straightening (Fig. 1, *b*), into the lumen of a No. 18 or 20 gauge transfusion needle in such manner that the barb of the hook protrudes over the edge of the needle, as shown in the accompanying figures (Fig. 1, *c*). About 1.5 or 2 cm. of the shaft of the hook lies within the lumen of the needle and if a slight bend is made in this shaft, it will retain its position within the lumen but can be removed for cleaning. When such a needle is inserted into the heart, or into a large vessel, the barb catches and prevents its coming out. In this way the labor, time, and trauma caused by the insertion and ligation of a cannula are avoided.

A large pressure flask (about 5 liters) is tightly stoppered and connected by means of rubber tubing with three outlets (Fig. 2) to (1) a blood pressure pump, (2) an Erlenmeyer flask containing 500 to 1000 cc. of 0.6 per cent neutral red (Grübler,

vital nach Ehrlich) in 0.9 per cent sodium chloride solution, and (3) Erlenmeyer flask containing 500 cc. of Zenker's fixing fluid containing 15 per cent formalin and no acetic acid (Fig. 2). The flasks containing neutral red solution and the fixing fluid each has a glass tube extending to its bottom, in order that all the fluid can be pumped out when an increased pressure in the flask obtains. These glass tubes

FIG. 1. Diagram illustrating construction of special cannula for perfusion of small animals through the heart.

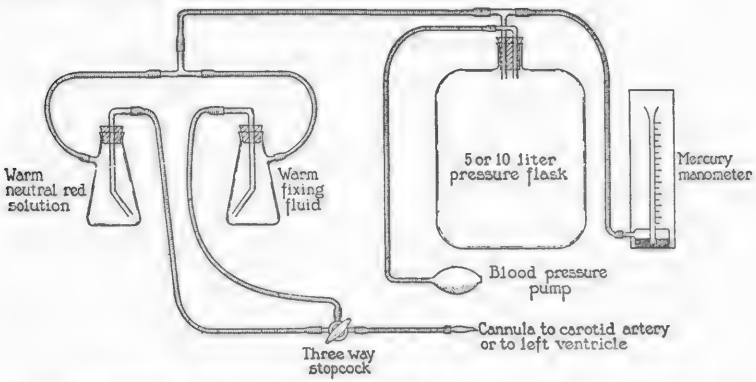
Warm neutral red solution
Warm fixing fluid
5 or 10 liter pressure flask
Mercury manometer
Blood pressure pump
Cannula to carotid artery or to left ventricle
Three way stopcock

FIG. 2. Apparatus used for staining of tissues and fixation of supravital dyes in cells of the entire body.

are connected by about 1.5 meters of small rubber tubing to two of the outlets of a three way stopcock. The remaining outlet of the stopcock is connected by a short rubber tube to the barbed cannula, as above described.

Staining of Tissues with Neutral Red.—An animal (rabbit, rat, guinea pig) is anesthetized with ether and fastened, ventral side up, with cords to an animal board. A jugular vein is exposed and ligated at the end proximal to the heart. An ordinary cannula is inserted into the distal segment. The animal should now

be under deep anesthesia. Two routes may be used for injection of the neutral red. One can either insert a cannula into a common carotid artery, ligating the distal segment of the artery, and inject the neutral red through the carotid artery into the aorta, or a window about 2 cm. square can be quickly made in the lower left chest near the sternum, care being exercised not to injure the lungs. With a pair of blunt forceps, the parietal pericardium is torn away, the apex of the heart held, and the special self-retaining cannula quickly inserted into the left ventricle and the neutral red solution allowed to pass into the heart, under a pressure of from 100 to 150 mm. of mercury. It is understood, of course, that the injection system must be free of air. At the moment of injection, the cannula in the jugular vein is opened and the blood and neutral red solution are allowed to escape. The cords binding the legs are immediately released to promote a ready circulation through the entire vascular system. The perfusion proceeds for from 15 to 25 minutes, at the end of which time the entire animal is deep red in color. The perfusion fluid should be warmed to from 38° to 40°C. and the rubber tubing carrying the fluid immersed in a vessel of warm water at this temperature. If the heart becomes greatly distended, an incompetency of the mitral valve results with a back flow of neutral red into the lungs through the pulmonary veins and considerable fluid may thus escape through the trachea into the mouth. This may be avoided by clamping the trachea with a hemostat or by clamping off the left auricle. Likewise, intercostal or internal mammary arteries may be clamped off if excessive fluid is being lost.

The amounts of fluid described above are for rabbits. For animals of smaller or larger size, the amounts can be more or less proportionate. Considerably less neutral red solution is required if one injects by way of the carotid artery instead of through the left ventricle. In the rabbit or larger animals, the injection through the carotid is the method of choice. With rats, mice, and all small animals, the route through the left ventricle is much more feasible and it is for these smaller animals that the special barbed cannula is especially adapted.

Fixation of Neutral Red in Tissues.—After all of the neutral red has been passed through the circulation, the stopcock is adjusted so that the fixing fluid warmed to body temperature is injected immediately following the neutral red and through the same channels. About 500 cc. of fixing fluid perfused over a period of from 10 to 20 minutes will reach every organ. The cannulae and all instruments are then immersed in running water to prevent tarnishing with mercury. Instruments made of nickel-steel are not as readily injured by the fixing fluid. Organs are now removed and small blocks placed in a vessel or vessels containing the fixing fluid, where they are allowed to remain for from 12 to 24 hours at room temperature.

Dehydration and Embedding.—It is necessary that blocks be cut not over 2 mm. in thickness, in order that rapid dehydration can take place. Such blocks are dipped in tap water and blotted to remove the excess of fixing fluid. They are then carried through the following solutions, blotting gently after each step up to No. 7.

Solution 1.	Absolute ethyl alcohol—rinse for 1 minute		
" 2.	" " "	5	minutes
" 3.	Pure acetone	20	"
" 4.	" "	20	"
" 5.	" "	20	"
" 6.	Xylol	20	"
" 7.	"	20	"
" 8.	56°C. Paraffin	1–2	hours
" 9.	56°C. "	1–2	"

The blocks are then embedded in 56°C. paraffin.

Staining.—The sections are cut from 4 to 10 microns in thickness, mounted on slides previously prepared with albumin fixative, and allowed to dry in a 37° incubator for 4 hours, or preferably over night. The sections on the slides can be excellently treated by the drop bottle method as follows: (1) Xylol to remove paraffin 10 to 20 seconds; (2) Absolute alcohol 5 seconds; (3) 95 per cent alcohol 3 seconds; (4) 95 per cent alcohol containing 1 per cent iodine 15 seconds; (5) 95 per cent alcohol several washings; (6) Absolute alcohol 5 seconds; (7) Clear in xylol; (8) Mount in balsam.

With the above method the macrophages, monocytes, and other vitally stained cells will stand out beautifully with the identical appearance of these cells, as seen in supravitally stained films of living cells.

An excellent counterstain can be employed after step (5) as follows:

(6 a) Distilled water for just long enough to overcome surface tension (3 to 5 seconds); (7 a) Goodpasture's acid polychrome methylene blue, full strength, for from 2 to 7 seconds; (8 a) Rinse quickly with water; (9 a) 95 per cent alcohol 5 seconds; (10 a) Absolute alcohol 5 seconds; (11 a) Xylol to clear; (12 a) Mount in balsam. Other methylene blue solutions may be used, but Goodpasture's permits of rapid staining, does not obscure the neutral red, and causes no troublesome precipitates.

Another method which is probably better than any other for demonstrating and preserving the cytoplasmic structures in supravitally stained macrophages and monocytes, and at the same time giving a satisfactory nuclear stain and a beautiful staining of the reticulum-fibers is a modification of Foot and Mènard's (8) method for staining reticulum by silver impregnation. The method is carried out in exactly the same manner as described by Foot, except that the sections are not bleached. When stained in this manner, the neutral red is of course washed out or obscured, but all the cytoplasmic structures which are stained with neutral red become intensely black. The

monocytes, pre-monocytes, and macrophages show the typical characteristics, except that the cytoplasmic bodies are black. Detailed descriptions of these cells stained in the tissues by the above methods can be found in an accompanying paper (9).

SUMMARY

A simple, rapid method for staining all the supravitally stainable cells in the body, as in supravital preparations with neutral red, is described, together with a method for faithful preservation of the dye in paraffin sections. The essential points of the technique are, first, to secure the reaction of cells to neutral red which corresponds to the so-called supravital technique, involving the reaction of only those substances which respond to the dye while the cell is living; and second, to preserve the stain through the processes of fixation, embedding, and counterstaining.

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THE ORIGIN OF MONOCYTES IN CERTAIN LYMPH NODES AND THEIR GENETIC RELATION TO OTHER CONNECTIVE TISSUE CELLS

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PLATES 12 TO 15

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The purpose of this study was to determine the position of monocytes in certain lymph nodes and, if possible, to ascertain from what cells they arise. The writer (1) has shown in a previous communication that lymph nodes from different parts of the body exhibit marked differences in cytology and that developing monocytes may be found as normal constituents of all the lymph nodes of the rabbit, except the large mass lying in the mesentery of the intestine. At the time of the earlier communication, no reliable method had been found for demonstrating the relationship of monocytes within lymph nodes to other tissue elements. The method used in these observations, which has been fully described in another communication (2), gives true supravital staining of the cells, faithfully preserves this reaction in paraffin sections, and shows their relation to other cellular elements.

The origin of the monocyte has been a much debated question. Various theories to the effect that monocytes arise from myeloblasts, lymphocytes, endothelium, histiocytes, reticulo-endothelial cells, and from undifferentiated cells have been advanced. Each is supported by a certain amount of evidence. However, no clear cut experiments are recorded to show that under normal conditions any of these cells give rise to monocytes. The writer agrees with Maximow (3) that the development of monocytes in the blood-forming tissues—"with the exception perhaps of the red pulp of the spleen"—has never been demonstrated.

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Description of Cells

The protocols to follow will state briefly what kinds of cells have been encountered in various tissues and will describe their morphological relations to each other. It seems expedient, therefore, to describe in some detail the cytological characteristics of the types of cells under discussion with special reference to supravital staining with neutral red and Janus green.

Primitive Undifferentiated Cells (Fig. 10, "c").—These elements can also be called mesenchyme cells. They are undifferentiated and possess multiple potentialities for the formation of various mesenchymal tissues. Entirely unstained by the supravital method, they are pale cells, with a round or oval nucleus and moderate cytoplasm containing no vitally stained granules. They have been described and illustrated by Cunningham, Sabin and Doan (4), by Forkner (1, 5), and others. They may be found in any of the blood-forming organs and probably possess multiple potentialities. In lymph nodes they doubtless represent the stem cells from which lymphocytes, monocytes, macrophages, and fibroblasts are derived.

Lymphocytes.—Maximow (6) believes that lymphocytes are not definitive cells, but are the stem cells from which various other cells develop. In the opinion of others, they represent a definitive cell. They appear as small, intermediate, and large forms which take very little or no neutral red into their cytoplasm when stained supravitally. Their nuclei are usually round, but may be oval, indented, or even horseshoe shaped. The chromatin is characteristically in heavy splotches. These cells usually have in their clear, hyalin cytoplasm a few round, globular, refractive, neutral red bodies. These bodies may at times be numerous and may even be arranged in the bay of the nucleus, in a rosette fashion about the centrosphere. However, the character of the neutral red granules, the character of the mitochondria, the type of motility, and the other general characteristics of the cells do not permit of their confusion with monocytes. Lymphocytes possess abundant mitochondria in their cytoplasm. These structures in themselves are of a different morphology than those in monocytes. They are larger and coarser. The mitochondria are apt to be in clumps near the nucleus, but at times are found scattered through the cytoplasm. The lymphocyte possesses quite a characteristic type of motility. It moves with the nucleus in the forward portion of the cell, as has been shown by Sabin (7).

Monocytes. (Figs. 1, 2, 4, 5, 6, 10).—Other terms which have been applied to these cells are: large mononuclears and transitionals of Ehrlich, endothelial leucocytes, and blood histiocytes. When stained with neutral red and Janus green, the monocytes of the rabbit possess features which permit of their easy differentiation from all other elements in the blood or tissues. They are large in size, as a rule, often exceeding all of the other blood cells in this respect. The

nucleus is often round or indented, more frequently kidney or horseshoe shaped. It possesses a delicate chromatin structure. The cytoplasm is not clear and hyalin, as in lymphocytes, but presents a foggy, or ground glass appearance. Within the cytoplasm are many neutral red bodies, the so-called "segregation apparatus." As Simpson (8) and Sabin (7) have shown, these bodies are, as a rule, arranged in a characteristic pattern, forming a rosette about the centrosphere. It is not uncommon, however, to find a diffuse distribution of these bodies in the cytoplasm. The neutral red bodies in monocytes possess a different shade of color from those in lymphocytes and also a different refractive power. They are, for the most part, non-refractive and are a salmon or brick red color. The mitochondria in monocytes generally appear much smaller than in lymphocytes and, as a rule, are more widely distributed. When a well-marked rosette is present, the mitochondria are frequently more numerous, or at least are better seen, at the periphery of the rosette. These cells have been illustrated by Simpson (8), Sabin (7), Forkner (1), and others.

Pre-Monocytes (Figs. 1, 2, 6, 7, 10).—This is a new term which is proposed for the purpose of designating one of the stages in the development of the monocyte. Prior to the present communication, no one has satisfactorily demonstrated the relations of monocytes to their precursors, under normal conditions, in any of the blood-forming organs. The demonstration in certain lymph nodes of the developmental cycle of the monocyte makes it necessary that a term be used to designate the stage of this cell intermediate between the monoblast and the mature monocyte. The pre-monocyte is characterized by the fact that it is constantly found in all the lymph nodes of rabbits, except in the large mass of lymphoid tissue lying in the mesentery of the intestine (Forkner, 1). It is similar to the adult monocyte of the blood in that it possesses somewhat the same morphological and biological characteristics when seen in supravital preparations. It differs from the adult cell in that the segregation apparatus is less well-developed and that it does not normally, except in rare instances, occur in the blood. The premonocyte is generally somewhat larger than the adult monocyte. Its nucleus is, as a rule, oval and is very faintly stained with the usual histological methods. The chromatin network is less well-developed than in the adult cell. The segregation apparatus consists of from one to several rows of very fine, non-refactive granules which may be arranged around a centrosphere or may be closely packed together in the center of the cell, often obscuring one side of the oval or round nucleus. These neutral red bodies are always in the region of the centrosphere and are never diffusely scattered throughout the cell. The mitochondria are abundant and are identical in morphology with those seen in mature monocytes. Doan and Sabin (9) described the maturation of monocytes in tuberculosis, using the terms monoblast, young monocyte, and monocyte. Their young monocyte corresponds to the pre-monocyte, except that they describe monoblasts and young monocytes in the bone marrow as having deeply blue basophilic cytoplasm as seen in fixed sections. The monoblasts and pre-monocytes which the writer has seen in lymph nodes do not possess a deeply basophilic cytoplasm when stained either with hematoxylin and eosin, or

with Giemsa stains. Their cytoplasm is very lightly stained and presents a very pale appearance

Monoblast (Fig. 10).—This term has been frequently used in the literature to designate the precursor of the monocyte. Bloom (10), however, in a recent paper, has made the statement that monoblasts do not exist. The demonstration in lymph nodes of monocytes in all stages of development allows one to shift the term monoblast from a more or less theoretical name to its proper place as a term designating that particular cell which is derived from a primitive undifferentiated cell and which is the precursor of the pre-monocyte. It is identified in preparations from peripheral lymph nodes by the fact that it is found in close association with pre-monocytes and monocytes in clumps of these cells. It appears to be identical with the pre-monocyte, except that it possesses no demonstrable segregation apparatus, that is, no supravitally stained neutral red bodies. It is easily demonstrable in paraffin sections of supravitally stained lymph nodes (Fig. 10).

Mesenchymal Macrophages.—There is no group of cells which has received more names, nor about which there is more confusing terminology than the cellular elements included under this term. The various terms present in the literature to designate the phagocytic cells under discussion are: macrophage, clasmatocyte, adventitial cell, resting wandering cell, rhagiocrine cell, reticular cell, reticulo-endothelial cell, endothelial leucocyte, pyroll cell, and histiocyte. The writer(11) has recently critically reviewed the literature on this subject and has proposed that we revert to the original descriptive term "macrophage" which was first applied to these cells by Metchnikoff (12, 13). It was proposed to limit the term, conforming to our present knowledge, and to include only those cells of mesenchymal origin which possess the property of phagocytosis of débris, or of staining intensely with moderate doses of vital dye in the living state. Where particular groups of macrophages are to be discussed in this paper, they will be designated as: macrophages of the lymphoid tissue, macrophages of the adventitia, macrophages of the common connective tissue, macrophages lining the lymph sinuses, etc. Such cells, supravitally or vitally stained, have been abundantly illustrated in the literature by many investigators (14, 15, 8, 16, 1). They are cells of large size (Figs. 4, 9). The nucleus is usually near the center of the cell, is round or oval, and has a delicate chromatin network. The cytoplasm is abundant and possesses few or many irregularly sized and shaped bodies, scattered throughout without any pattern or definite arrangement. These bodies may represent débris which the cell has phagocytized, or in many instances, they represent vacuoles in which the neutral red or vital dyes have been assembled. The cytoplasmic structures are not only heterogenous in size and shape, but also frequently show various shades of staining. This is in contrast to the uniform homogeneous appearance of the cytoplasmic structures in monocytes. The macrophages lining the sinuses (reticulo-endothelial cells—Fig. 8) are similar in their reactions to dyes and foreign material. The latter cells are fixed, are often flattened or elongated, and can be selectively stained with vital dyes. By appropriate experiments, reported in the protocols of this

paper, it is shown that these cells are among the first to stain with vital trypan blue, whereas the phagocytic macrophages of the reticular syncytium and the free macrophages in the lymphoid tissue may be almost entirely unstained with few doses of this dye. However, this difference is probably dependent on the fact that the former, by virtue of their anatomical position, are more directly exposed to the material passing through the lymph node. It is also significant that the macrophages lining the lymph sinuses (reticulo-endothelial cells) and the phagocytic macrophages of the reticular syncytium are identified with the formation of reticulum fibers, whereas free macrophages are not.

Plasma Cells.—These cells should be described here because they frequently are found in lymph nodes of rabbits and other mammals. So far as the writer is aware, there has been only one description of these cells as they appear in supravital preparations. An analysis of the literature on this point will be considered in the discussion at the end of this paper. On the whole, they are about the size of intermediate lymphocytes, but may be smaller and are often somewhat larger. They possess, as a rule, more cytoplasm in relation to the nucleus than do lymphocytes. The nucleus is generally round or slightly oval, and is often eccentrically placed in a manner which is well-known for these cells. The one special characteristic possessed by these cells, when seen in supravital preparations stained with neutral red and Janus green, is that the cytoplasm has a peculiar and characteristic appearance. It is homogeneous and possesses a faint yellow tinge, almost like that of a nucleated red cell in which the full quota of hemoglobin is not present. Because of this fact, plasma cells in lymph nodes have often been mistaken for developing erythrocytes. Plasma cells in lymph nodes often contain in their cytoplasm, as do lymphocytes, one or a few small, refractive, globular, neutral red bodies. Mitochondria are frequently numerous and of the same type as those described for lymphocytes. In no instance have plasma cells been seen possessing segregation granules resembling those found in monocytes or pre-monocytes. They are easily distinguished from these latter cells.

Materials and Methods

Normal rabbits, guinea pigs, and rats vitally stained with trypan blue have been used in these experiments. The method has been completely described in an accompanying paper (2) which should be consulted for repetition of these experiments. Let it suffice to say here that sections of tissues which have been stained supravitally alone or in combination with vital staining are in many instances more instructive than the living supravitally stained cells. The chief advantage of the method is that the supravital staining is preserved in paraffin sections. A few typical protocols of experiments are given in the following pages.

PROTOCOLS OF EXPERIMENTS

Experiment 1.—Rabbit 2. The animal fresh from stock was etherized. Neutral red, 0.6 per cent, in 0.9 per cent sodium chloride solution, dissolved by warming and then filtered, was injected at body temperature and at 150 mm. of mercury pressure, through a cannula into the exposed left ventricle. A cannula was placed in the right jugular vein, through which the perfusion fluid and blood were allowed to escape. The animal was thus perfused with about 800 cc. of the neutral red solution over a period of 25 minutes. Immediately following, Zenker-formol solution was similarly injected at body temperature through the same cannula, over a period of 20 minutes. Tissue from the various organs was removed, placed in fixing fluid of the same kind, and allowed to remain over night. The method of staining, fixation, dehydration, embedding and counterstaining is described in detail in the accompanying paper (2).

The organs were all well stained except the bone marrow, which contained only a few areas of stained cells in the immediate neighborhood of the nutrient arteries. Much neutral red had accumulated in the stomach and intestines which were dilated and the contents of which were dark red in color.

Peripheral lymph nodes were normal in size and stained a dark red color. On histological examination, all the nodes were essentially alike, except that some contained more monocytes than others. Some of the lymph nodes were heavily laden with monocytes which were situated in the lymphoid tissue in and around the primary follicles. Also in some nodes monocytes were present in large numbers in certain of the secondary follicles, the so-called germinal centers. Most of the germinal centers, however, contained very few or no monocytes. The monocytes, as seen in sections, were often in clumps of from four to twenty or more cells (Figs. 1, 3, 5, 6, 10). These clumps were characteristically a part of the parenchyma of the lymphoid tissue and were not free or separated from the lymphoid cells by any perceptible boundary. Many monocytes were also found diffusely scattered through the cortical tissue, but were almost never present in the medullary cords. These cells were easily recognized by their characteristic appearance. It is interesting to note that they were not in any way related, in their position, to cells lining the sinuses. It is true that occasional monocytes are found in the sinuses (Fig. 5) and in the medullary cords, but this is not at all a common occurence. Their histological appearance cannot be confused with that of the macrophages lining the lymph sinuses (Fig. 8), the cells of the so-called reticulo-endothelium. Monocytes in various stages of development can be easily demonstrated, particularly in areas where monocytic cells are numerous (Fig. 10). The life cycle can be traced from undifferentiated cells through the stages of monoblasts and pre-monocytes to mature monocytes.

Free macrophages, so-called reticular cells, are likewise numerous in the peripheral lymph nodes, but are not as abundant as monocytes. They are found within the sinuses, in the parenchyma of the lymphoid tissue, and may be scattered through the follicles (Figs. 4, 9), being present at times in the secondary follicles.

These free macrophages vary tremendously in size from that of a large lymphocyte to giant cells with abundant cytoplasm and several nuclei. As a rule the cytoplasmic bodies are scattered throughout the cell without pattern and conform closely to the description previously given. However, certain unmistakable, free macrophages have the cytoplasmic bodies arranged in the form of a very large rosette. This rosette is not composed of the uniform, fine, brick red, non-refractive, neutral red bodies typical of the monocyte, but, on the contrary, the vacuoles are coarse, irregular, of different shades of color, and possess different refractive properties. By diligent search, cells can be found which cannot with certainty be placed in one or the other group. These cells must be considered carefully, for they suggest that under physiological conditions monocytes may be transformed into macrophages. This point will be considered in the discussion to follow.

Plasma cells were found in the lymph nodes of this animal (Rabbit 2). In some areas they were numerous. These areas were chiefly located in the less dense lymphoid tissue and not in the follicles themselves. The plasma cells were easily distinguished from all the other cellular elements.

Mesenteric lymph nodes differ considerably in their cytology from the other lymph nodes, as discussed in detail in an earlier paper (1). The essential differences are: (a) almost complete absence of monocytes in mesenteric lymph nodes, but an abundance in peripheral lymph nodes; (b) presence of more macrophages, chiefly those lining the sinuses, than in peripheral nodes; (c) presence of many more large lymphocytes with abundant basophilic cytoplasm in the mesenteric than in the peripheral lymph nodes.

Spleen.—No developing monocytes were found. Occasional, fully developed monocytes were present. Many free macrophages were present, some of which contained fragmented cellular débris.

Thymus.—An abundance of free macrophages was present, chiefly limited to the cortical zone. No monocytes could be found in either the cortical or medullary zone.

Liver.—No monocytes were present. Many macrophages (Kupffer cells) were in the capillaries. The organ presented an entirely normal appearance.

Omentum.—Paraffin sections demonstrated a few small groups of monocytes, and this possibly represents a place of development for these cells. Their number, however, was insignificant when compared with the number found in the lymph nodes other than the mesenteric mass of lymphoid tissue. Many monocytes appeared to lie on the surface of the serosa, as though they had been present in the peritoneal fluid and had adhered to the omentum. It must be stressed, however, that in supravital films of the omenta of normal rabbits, rats, and guinea pigs, milk spots can be seen composed almost exclusively of monocytes, others containing primarily macrophages, others lymphocytes, and still others primitive undifferentiated cells. Such findings indicate that in the omentum there are present many cells possessing mesenchymal potencies. Many free macrophages were found in the omentum of the animal described in this protocol. Also, many elongated,

fixed macrophages in close association with fibroblasts and connective tissue fibers could be demonstrated.

Bone marrow.—The bone marrow was not well stained in this animal but will be described in some of the protocols which follow.

Many other organs were studied, but their description is not relevant.

Experiment 16.—Rabbit 16. The animal was used in an attempt to inject neutral red more directly into the vessels of the posterior extremities in order to stain supravitally the bone marrow cells. The abdomen was opened under ether anesthesia and a cannula inserted into the abdominal aorta, just above the pelvic brim. Neutral red, 500 cc. of a 0.6 per cent solution in normal saline, was then slowly perfused through the vascular system of the posterior extremities under the same conditions as those in Experiment 1. The same fixing fluid was also used.

After this process the bone marrow was removed intact with blunt forceps. Dehydrating, clearing, and embedding were carried out as in Experiment 1. The marrow was distinctly red, particularly in the neighborhood of the nutrient vessels. Lymph nodes from the popliteal and inguinal groups were also saved.

Microscopically the marrow showed large areas which were ideally stained with neutral red in a manner which renders the differentiation of the cellular types easy. The bone marrow from the long bones of rabbits is very active in blood formation and contains granulocytes, erythrocytes, and megakaryocytes in all stages of development. Macroscopically it is red and highly vascular. It is not within the scope of this paper to describe the histological details of the findings, except to say that monocytes cannot be found in any part of the marrow. Free macrophages are scattered sparingly in the tissue. Macrophages lining the capillaries in the marrow, the so-called reticulo-endothelial cells, are abundant and for the most part are elongated and contain coarse, neutral red bodies in their cytoplasm. They resemble the macrophages lining the lymph sinus in lymph nodes. In no instances can cells be found which can be interpreted as transitional phases between macrophages and monocytes.

It had been demonstrated in the above experiments that all supravitally stainable cells in the body could be easily stained with neutral red and the color preserved in paraffin section. Vital staining with dyes such as trypan blue, Niagara blue, and lithium carmine has long been employed to mark out the cells of the so-called reticulo-endothelial system. It was proposed, therefore, at the suggestion of Professor Aschoff, to use the combined methods of supravital staining with neutral red and vital staining with trypan blue to determine whether or not the developing monocytes really belong to the reticulo-endothelial system, the system of histiocytes, or of mesenchymal macrophages. Paraffin sections of such tissues show that the cytoplasmic structures in the cells which have been previously vitally stained with trypan blue and subsequently counterstained with neutral red present a violet color, whereas those cells which have not been capable of taking up trypan blue and contain only neutral red possess a pure red color, just as though no trypan blue had been introduced. The following experiment was performed.

Experiment 17.—Rabbit 17. A normal rabbit was given a series of five intravenous injectons of a sterile, 1 per cent, aqueous solution of trypan blue. The injections were given every second day. 24 hours after the last injecton of trypan blue, the animal was perfused, through the left heart, with 1000 cc. of 0.5 per cent neutral red, dissolved in normal salt solution. The perfusion and fixation were carried out under the same conditions as in Experiment 1. The general appearance of the tissues changed from a blue to a purple color during the course of the perfusion with neutral red.

Microscopic examination of the lymph nodes demonstrates clearly that the pre-monocytes and monocytes in the lymph nodes are not stained with trypan blue. On the other hand, many free macrophages (reticular cells), many phagocytic cells of the reticular syncytium, and practically all of the macrophages lining the lymph sinuses (reticulo-endothelial cells) contain an abundance of trypan blue, as well as neutral red. It must be stated that many macrophages in the lymphoid follicles contain very little or no trypan blue. Also, certain cells can be seen to be more abundant than in normal animals, which morphologically represent stages between typical monocytes and typical macrophages and which are, almost without doubt, transition phases in the development of the latter from the former. These cells are likewise intermediate in their reaction to trypan blue, taking only a small amount of this dye. All transition phases can be demonstrated between monocytes containing neutral red but no trypan blue, and macrophages containing an abundance of both colors.

Another experiment was undertaken to confirm these findings. It was proposed to inject much more of the trypan blue to determine whether excessive doses of this dye would alter the above findings.

Experiment 18.—Rabbit 18. This animal was treated with five intravenous injections of 6 cc. each of a 1 per cent aqueous solution of trypan blue, followed by a series of four injections of 8 cc. each of the same solution. These injections were also given on every second day. 24 hours after the last injection, the animal was perfused with 800 cc. of 0.7 per cent neutral red solution and then with fixing fluid, as in the previous experiments.

The results of this experiment are, so far as the point under discussion is concerned, in entire agreement with those of Experiment 17. There are, however, a few additional points which should be mentioned. It will be remembered that in Experiment 17 the macrophages lining the lymph sinuses were strikingly colored with the trypan blue, whereas only a portion of the free macrophages and macrophages of the reticular syncytium were stained. In Experiment 18, practically all of these macrophages contained an abundance of trypan blue,

whereas monocytes lying in close proximity to these cells remained unstained with vital dye, but were richly colored with neutral red (Fig. 9). This fact demonstrates that, even with prolonged administration of large doses of vital dye, the monocytes and pre-monocytes of the lymph nodes remain unstained. In this experiment there were more cells which might be interpreted as intermediate stages from monocytes to macrophages (Fig. 9).

It is interesting also in this experiment that the macrophages of the liver capillaries (reticulo-endothelial or Kupffer cells) were increased considerably in number and for the most part contained a large amount of trypan blue. Also in the sinuses, one sees occasional typical monocytes, often three or more in a clump. Moreover, typical transition forms from monocytes to macrophages can be demonstrated. This finding in the liver will be discussed in another portion of this paper.

DISCUSSION

The literature on the origin of the monocyte shows that no one has as yet proved the derivation of this cell from any fixed cell in the body. Numerous investigations have been reported on the mode of production of these cells under pathologic or tissue culture conditions. These reports are contradictory, excellent workers stating that they arise from endothelium, lymphocytes, myeloblasts, histiocytes, etc., and equally competent investigators denying these statements. The report contained in the present contribution deals with normal animals and demonstrates a site of origin and development of monocytes under physiological conditions. It shows that monocytes are formed in certain specific blood-forming organs and in this respect are analogous to all the other structural elements of the blood. The analogy can be carried even further for, like other white blood cells, they are shown to be derived from primitive, undifferentiated, cellular elements and their various stages of development can be clearly demonstrated.

It is not denied that monocytes may, under abnormal conditions, be abnormally produced in abnormal locations. On the contrary, it has been shown conclusively by numerous investigators that under experimental or pathologic conditions, monocytes may arise in various organs and tissues of the body. This, however, is likewise true of lymphocytes and granulocytes.

The methods here reported for the study of the development of

monocytes in lymph nodes have been equally applicable to the study of these and related cells in all organs of the body. They have demonstrated conclusively that, in the bone marrow of the long bones of rabbits, developing monocytes are not to be found, although the marrow is very active for the production of granulocytes and erythrocytes. This confirms the observations of Sabin and Doan (17). These authors gave the first direct, conclusive evidence that in the rabbit, under physiological conditions, monocytes are not derived from myeloblasts or any other cell in the bone marrow.

Monocytes have been said to arise in considerable numbers in the spleen. Sections of supravitally stained spleens of the animals used in these experiments have failed to show more than occasional monocytes and very rarely pre-monocytes. It is thus apparent that the spleen plays no significant part as an organ in which monocytes are produced.

Maximow (3), Bloom (10), Masugi (18), Rhoads and Parker (19), Witts and Webb (20), and others have maintained that monocytes do not occur in any significant numbers in lymph nodes. All of these investigators employed the supravital technique, together with other methods, for their studies. Maximow (3) and Bloom (10) have used these results as one of their reasons for affirming the original contention of Maximow that monocytes are formed from lymphocytes in the blood stream by individual transformation of the latter cells. On the other hand, Masugi (18) made use of the same evidence to uphold his view and that of Aschoff (21) that monocytes are formed by transformation from histiocytes and reticulo-endothelial cells. It has been possible to utilize the same evidence for the support of each view because a gap has been present in our knowledge of the development of monocytes.

Although Bloom (10) recently has reported that all transition stages between lymphocytes and monocytes may be found in the blood of animals experimentally infected with *B. monocytogenes*, this has not been confirmed by Witts and Webb (20). Furthermore, no cells are shown in Bloom's figures, or described in his paper, which correspond to the early developmental forms of monocytes, as seen in peripheral lymph nodes. It is not intended to imply that the development of monocytes from lymphocytes in the blood stream is impossible, but to indicate that the evidence is insufficient to prove that under physiological conditions this is the mechanism for their formation.

In other experiments of Bloom (22, 23) he cultured lymph from the thoracic duct and after a time recovered monocytes. But one cannot be certain that all the cells in the thoracic duct are lymphocytes. It is not uncommon to find undifferentiated cells which possibly possess multiple potentialities. Even if one assumed that a pure culture of lymphocytes could give rise to a pure culture of monocytes, one would still have to show that this is the mechanism by which monocytes are produced under physiological conditions.

None of my evidence supports the view that monocytes are, under normal conditions, derived from ordinary blood vessel endothelium, macrophages lining the lymph sinuses (reticulo-endothelium), or from free macrophages. Undoubtedly however, desquamated reticulo-endothelial cells do occur under certain conditions in the blood stream, as pointed out by Mallory (24), and many others. Sabin and Doan (25) maintain that under normal conditions there is a practically constant desquamation of endothelial cells into the circulating blood in rabbits and man. It is difficult to conceive of how these cells could give rise to monocytes. Furthermore, no direct evidence for such transformation is recorded in the literature. The reports of Schilling (26) and of others that monocytes have been seen to arise from the Kupffer cells in the liver demand discussion, since it has been shown in this communication (Experiment 18) that monocytes may be found in the liver capillaries in small numbers when animals are vitally stained with trypan blue. In addition, transition forms between monocytes and macrophages and between monocytes and epithelioid cells are demonstrable. It is probably such findings in pathological material that have been responsible for the theory that monocytes arise from Kupffer cells. The evidence, as will be pointed out presently, is decidedly against the theory of the transformation of Kupffer cells into monocytes.

On the other hand, one does find evidence for the hypothesis that monocytes may be transformed into free macrophages, and this probably represents one of the modes of formation of the latter cells. Such proof is recorded in the protocols of this paper. It is consistent with Simpson's (8) production of macrophage showers in the circulating blood. These showers were generally preceded by a marked monocytosis and the presence of cells which probably represented transition phases between the two types of cells. A typical protocol shows that the shower phenomenon first occurred after twenty-six injections over a period of 2 months. Masugi (18) has recently contested the findings of Simpson (8), although he seems not to have repeated the experiments. He explains the macrophage showers of Simpson as due to aspirated pericardial fluid.

Tissue cultures of the buffy coat of the blood (27, 28) have likewise shown that monocytes readily may be converted into macrophages. Carrel and Ebeling (29) compared the results of tissue cultures of monocytes from the blood and macrophages from the subcutaneous tissues of chickens. They found a transformation of monocytes into macrophages.

From the experiments recorded in this communication and from the experiments of many workers in the past, it appears to be proved that macrophages in lymph nodes may arise directly from the undifferentiated cells of the reticular syncytium, or that they may arise from monocytes which, in their turn, are derived from the same mother cells.

A schematic outline of the development of monocytes and macrophages in lymph nodes is presented in the accompanying diagram.

Diagram Representing Normal Development of Monocytes and Macrophages in Lymph Nodes

Common Endothelial Cell

(a) Lines ordinary blood vessels.
(b) Does not produce reticulum.
(c) Does not stain with vital or supravital dyes.
(d) Does not phagocytize débris.
(e) May desquamate into blood as transient inhabitant.

Fixed Macrophage

(Phagocytic cell of reticular syncytium)

(a) Produces reticulum fibers.
(b) Stains with supravital and vital dyes.
(c) Phagocytic for débris.
(d) Cytoplasmic structures have no characteristic pattern.

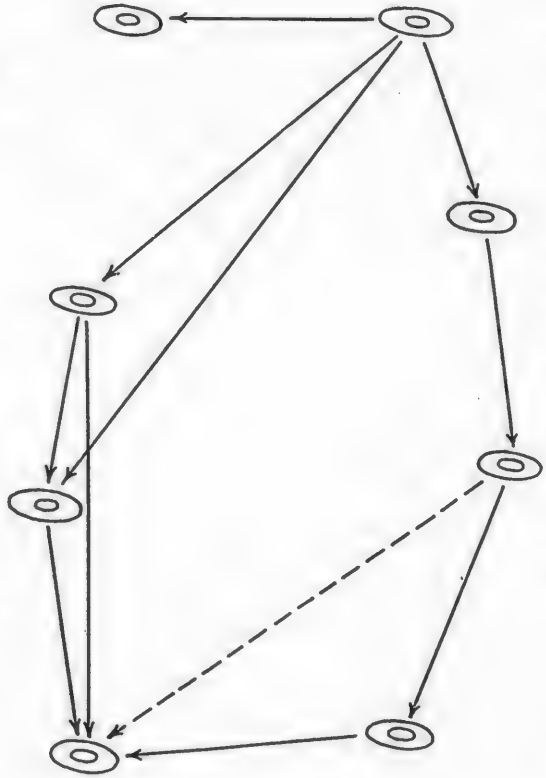
Macrophage Lining Sinus

(Reticulo-endothelial cell)

(a) Produces reticulum fibers.
(b) Stains with vital and supravital dyes.
(c) Phagocytic for débris.
(d) Cytoplasmic structures have no characteristic pattern, size, or shape.
(e) May become detached to form free macrophage.

Free Macrophage

(a) Does not produce reticulum fibers.
(b) Markedly phagocytic for débris.
(c) Stains with vital and supravital dyes.
(d) No pattern to cytoplasmic structures.
(e) Is a wandering cell.
(f) Occurs in blood as transient inhabitant.



Primitive Cell (Undifferentiated mesenchyme cell)

(Undifferentiated, non-phagocytic cell of reticular syncytium)

(a) May produce reticulum fibers.
(b) Contains no supravitally or vitally stainable granules.
(c) Possesses multiple potentialities.

Monoblast

(a) May or may not be identified with production of reticulum fibers.
(b) Somewhat more differentiated than the primitive cell.
(c) Found in association with pre-monocytes and monocytes.
(d) Contains no vitally or supravitally stainable granules.
(e) Not found in blood.

Pre-Monocyte

(a) Does not form reticulum fibers.
(b) Somewhat more differentiated than monoblast.
(c) Found in association with monoblasts and monocytes.
(d) Contains the beginning of a rosette of neutral red bodies.
(e) Does not stain with vital dyes.
(f) Rarely found in normal blood.
(g) May be experimentally produced in blood.

Monocyte

(a) A fully differentiated cell.
(b) Contains many neutral red bodies, usually in form of a rosette.
(c) Is smaller than pre-monocyte.
(d) Is a normal constituent of the blood.

All the cells mentioned in the diagram may be seen in peripheral lymph nodes when stained and sectioned according to the writer's methods (2). They may originate in other manners besides those there given, but the diagram indicates one common method of their origin under physiological conditions. The peripheral lymph nodes are the only organs of rabbits in which one can constantly find monocytes in all stages of development.

McJunkin (30) has thought that monocytes are derived from what he calls "reticular lymph vessel endothelium" in lymph nodes. His methods, in my hands, have proved unsuccessful for the demonstration of monocytes, supravitally stained in paraffin sections of lymph nodes. On the other hand, many free macrophages and macrophages lining the lymph sinuses can be stained and demonstrated by his methods. Possibly McJunkin has confused these elements with monocytes.

What is the relation of monocytes to the reticulo-endothelial system? The experiments (Nos. 17 and 18) recorded in the protocols have demonstrated that monocytes and pre-monocytes, in the lymph nodes, remain unstained when the animals are repeatedly injected over a long period of time with trypan blue. These facts are in agreement with the general opinion that monocytes do not stain with vital dyes. There is one exception, however, which must be mentioned here. Doan, Sabin, and Forkner (31) have shown that some monocytes, epithelioid cells, and epithelioid giant cells may be stained vitally when directly exposed to trypan blue over a long period of time. This result might have been predicted, since Kiyono (14), Evans and Scott (15), and others, have demonstrated that in connective tissues even fibrocytes may become vitally stained after prolonged treatment with trypan blue or Niagara blue. The only conclusion which one safely can make with regard to the relation of monocytes to the reticulo-endothelial system is that they do not stain vitally by the usual methods which are used to demonstrate this system.

The question of the relationships between lymphocytes, monocytes and plasma cells in the rat has been discussed by Bloom (32). He has concluded that plasma cells often possess rosettes of neutral red bodies and that all transitions exist between plasma cells and monocytes in lymph nodes. The present work on rabbits does not support the conclusions of Bloom.

The question naturally arises: How do monocytes reach the blood stream, a point which has already been discussed by the writer (1). They probably gain admission to the capillaries by means of their own motility in much the same manner as do the granular leucocytes. It is true that in the peripheral lymph nodes developing monocytes are frequently found in close proximity to the capillary vessels (Fig. 10). It is also probable that many lymphocytes which develop in the spleen and lymph nodes wander into the blood stream.

SUMMARY

1. The theories for the origin of monocytes from myeloblasts, lymphocytes, endothelium, macrophages, and primitive cells are reviewed and considered.

2. Monocytes in all stages of development have been demonstrated to be present constantly in large numbers in all the lymph nodes of the body, except in the large mesenteric group.

3. The relations of these cells to undifferentiated cells, lymphocytes, macrophages, plasma cells, and endothelium are described.

4. The origin of adult monocytes from primitive undifferentiated cells through the stages of monoblasts and pre-monocytes is described and illustrated.

5. The demonstration in certain lymph nodes of innumerable monocytes in all stages of development permits of a shifting of the term "monoblast" from a more or less theoretical name to its proper place as a term designating that particular cell which is derived from a primitive undifferentiated cell and which is the immediate precursor of the pre-monocyte.

6. The term "pre-monocyte" is proposed to designate the intermediate stage between the monoblast and the mature monocyte.

7. Evidence is advanced to show that monocytes are an independent strain of cells, but that under physiological conditions they may be transformed into macrophages, this representing at least one way in which the latter cells normally are produced.

8. In no organs or tissues other than in certain specific lymph nodes, chiefly the peripheral group, can one constantly find monocytes in all stages of development.

9. Developing monocytes occasionally may be found in small num-

bers in the spleen, mesenteric lymph nodes, Peyer's patches, subcutaneous connective tissues, lungs, and omenta of normal rabbits, but their presence is by no means constant and their numbers are insignificant in comparison with those found in the peripheral lymph nodes.

10. Monocytes and pre-monocytes do not stain by the common methods used for the demonstration of the reticulo-endothelial system and therefore must be considered for the present as independent of this system, except in so far as monocytes may be transformed into macrophages.

11. Plasma cells, stained with the supravital technique, as seen in lymph nodes, are described. No basis has been found for the theory that plasma cells and monocytes are closely related structural elements.

I wish to express my appreciation and gratitude to Professor Ludwig Aschoff, in whose laboratory this work was done, for his many helpful suggestions.

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EXPLANATION OF PLATES

PLATE 12

FIG. 1. Peripheral lymph node of a normal rabbit. × 1000. Paraffin section. Stained supravitally with neutral red. Here is an area of developing monocytes in the less dense lymphoid tissue at the periphery of a primary follicle. This is not an isolated or single group of monocytes. The picture may be duplicated many times in almost any section of the peripheral nodes, when properly stained. All stages are present, from monoblasts to mature monocytes with well-formed rosettes.

FIG. 2. Peripheral lymph node of a rabbit. ×820. Paraffin section. Stained supravitally with neutral red. Counterstained lightly with Goodpasture's acid polychrome methylene blue. This figure shows developing monocytes within a primary follicle of the lymph node. The size of the cells can be compared with that of the lymphocytes at the left of the figure. The delicate, faint outlines of the nuclei can be clearly seen.

PLATE 13

FIG. 3. A follicle in a peripheral lymph node of a normal rabbit; a low power view of the same area shown in Fig. 2. × 280. Paraffin section. Supravitally

stained with neutral red. Lightly counterstained with Goodpasture's acid polychrome methylene blue. Not all follicles contain monocytes. Many contain more monocytes than this and some may be almost entirely composed of such cells. It will be seen that they are independent of reticulo-endothelium and of common blood vessel endothelium. They develop from the undifferentiated parenchymal cells of the stroma.

FIG. 4. Peripheral lymph node of normal rabbit. ×1200. Paraffin section. Stained supravitally with neutral red and counterstained by a modification of Foot and Menard's rapid silver method. The upper part of the photograph represents the peripheral portion of a primary follicle. In the center, at the periphery of the follicle, two monocytes with well-developed rosettes can be seen, "a." To the right is a free macrophage showing the character of the cytoplasmic structures in these cells, "b."

FIG. 5. Peripheral lymph node of a normal rabbit. ×1200. Paraffin section. Staining as in Fig. 4. Here monocytes and pre-monocytes are shown in a peripheral sinus. They rarely appear in the sinuses. They are shown here as free cells which have probably made their way into the sinus by means of their own motility.

FIG. 6. A group of monocytes, pre-monocytes, and monoblasts in a peripheral lymph node of a normal rabbit. ×1200. Staining as in Fig. 4.

PLATE 14

FIG. 7. Peripheral lymph node of a normal rabbit. × 1200. Stained as in Fig. 4. Here is shown a group of early pre-monocytes and monoblasts. The former have small rosettes not fully developed, whereas the latter are entirely similar, except for the absence of the neutral red bodies.

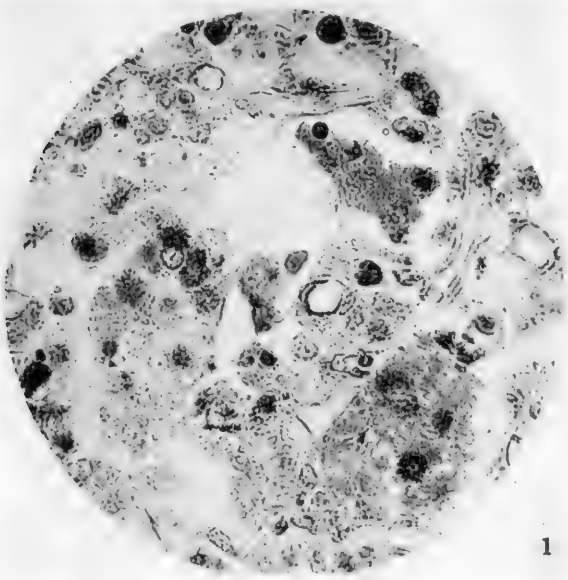
FIG. 8. An area of lymphatic tissue between follicles of lymphoid tissue of a normal rabbit. × 1200. Stained as in Fig. 4. The macrophages of the sinuses (reticulo-endothelial cells) are shown. These cells contain reticulum fibers and also irregular masses of neutral red. Monocytes cannot be found developing from these cells. Compare the monocytes of Fig. 7 with the reticulo-endothelial cells of Fig. 8.

PLATE 15

FIG. 9. Peripheral lymph node of rabbit treated with 9 doses of trypan blue (Experiment 18). Subsequently stained supravitally with neutral red. Paraffin section × 820. Here can be demonstrated intermediate stages in the transformation of monocytes into free macrophages. "a" points toward a cell which has a large rosette of neutral red bodies. In addition, a few globules of trypan blue have been taken into the cell. The granules of the rosette are larger than those usually seen in monocytes. It is a transition form but still possesses most of the characteristics of a monocyte. The cell to the right of the arrow leading from small letter "a" represents another step in the transition. It possesses an abundance of both neutral red and trypan blue. The cell indicated by "b" is a genuine macrophage which has probably developed from a primitive cell directly without first having

been a monocyte or pre-monocyte. It contains both dyes in abundance. "c" indicates a fully developed monocyte. It contains much neutral red, but no trypan blue. "d" indicates a developing monocyte, a pre-monocyte, with a characteristic rosette containing only neutral red bodies.

FIG. 10. An area of developing monocytes closely adjacent to a capillary blood vessel in a peripheral lymph node. Paraffin section × 820. Stained as in Fig. 1. The small letters "a" point to cells which are regarded as monoblasts. They do not possess rosettes and yet are somewhat more differentiated than the primitive cells "c." The letter "b" indicates a pre-monocyte and "d" a well-developed monocyte. At the upper right hand corner a small capillary blood vessel is seen.



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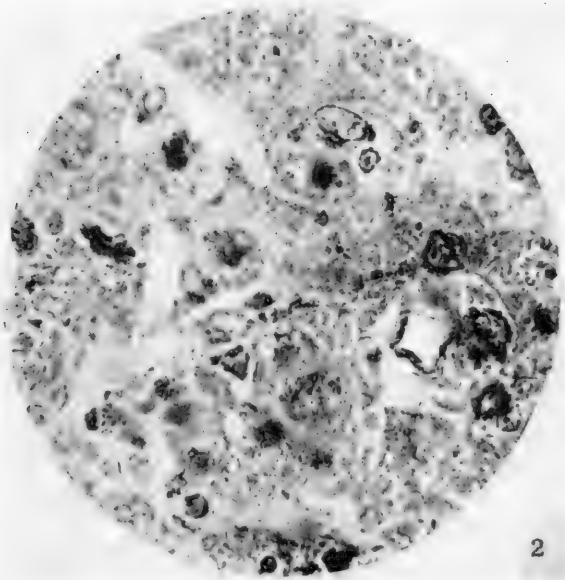

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(Forkner: Origin of monocytes in lymph nodes)

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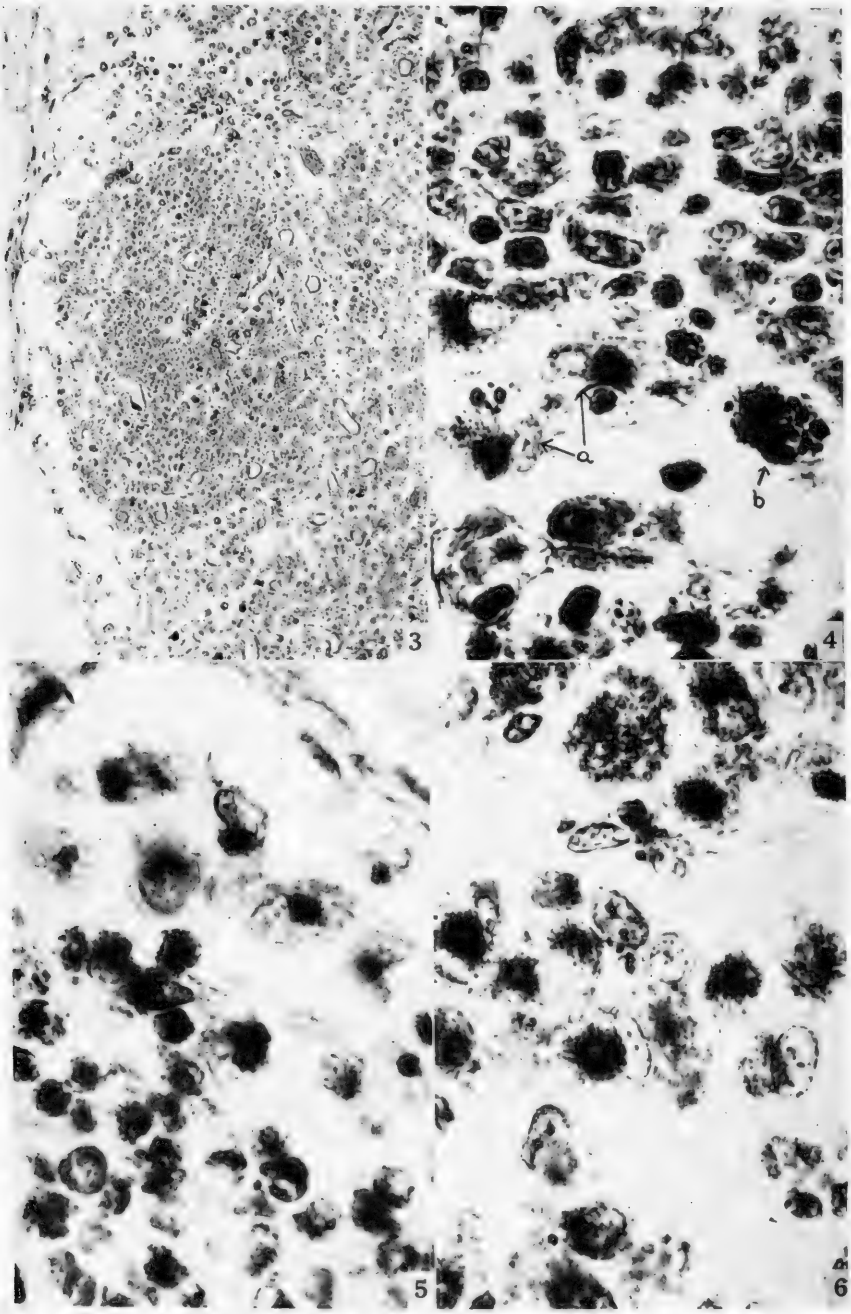

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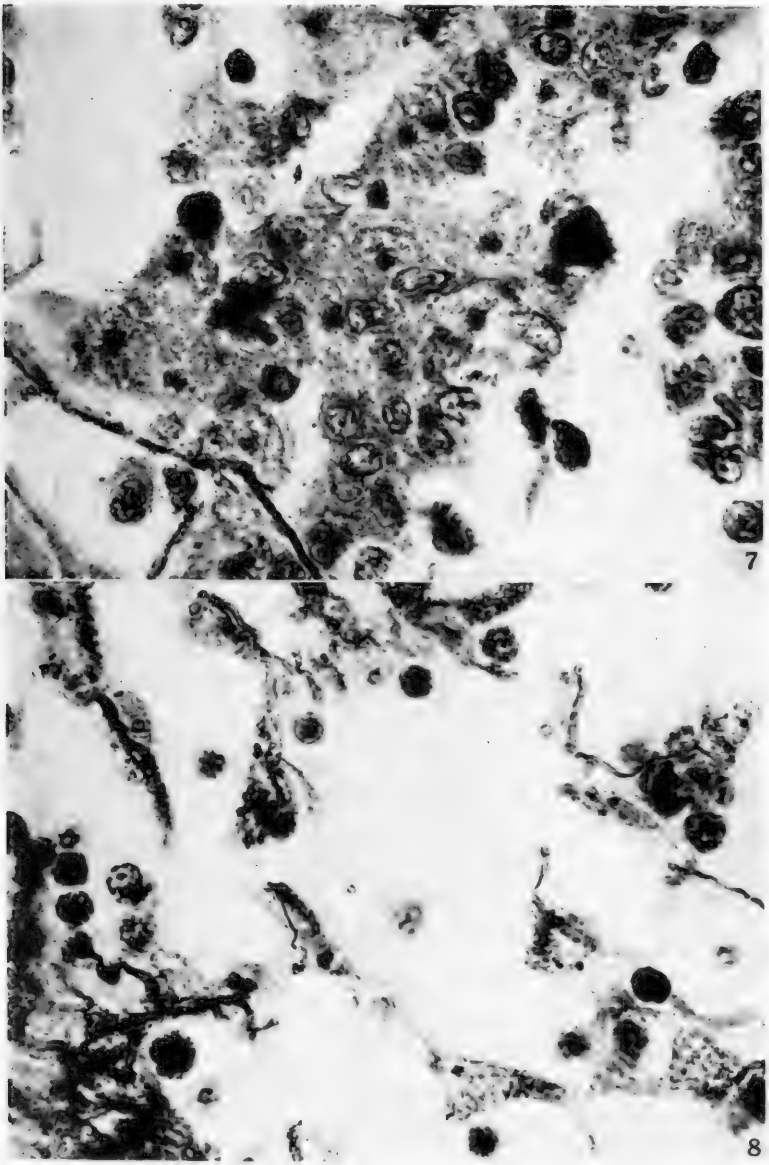

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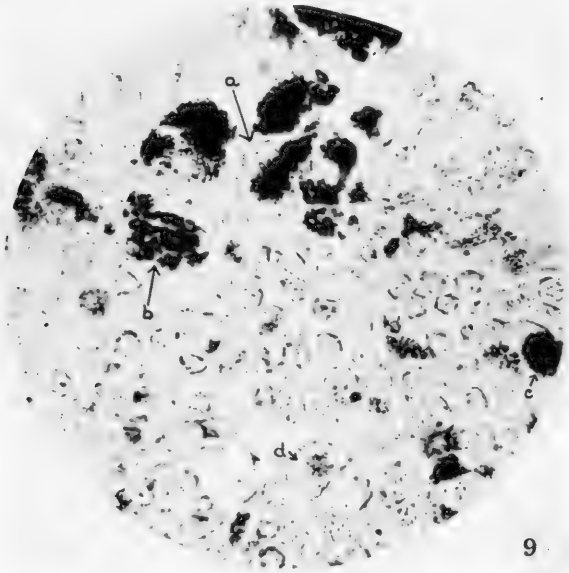

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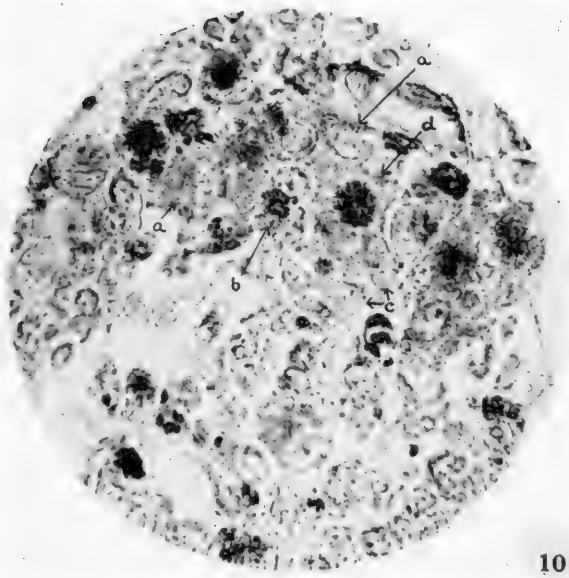

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(Forkner: Origin of monocytes in lymph nodes)

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THE SUSCEPTIBILITY OF MARMOSETS TO YELLOW FEVER VIRUS

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PLATE 16

(Received for publication, May 16, 1930)

Previous studies at this laboratory have demonstrated a relative susceptibility to yellow fever virus in the Capuchin (1, 2), "spider," "woolly," and "squirrel" (3) monkeys of Brazil. In the present paper it is proposed to discuss transmission experiments with certain more primitive genera, the members of which are usually called "marmosets" in English and "saguins" in Portugese. These are very small and rather delicate monkeys, not particularly suitable for laboratory experimentation. It has been our experience that the mortality is rather high among recently captured specimens, but that the survivors which become accustomed to captivity can be used after a few weeks with less likelihood of deaths from unexplained causes during the course of the experiments.

Stokes, Bauer, and Hudson (4) inoculated marmosets with yellow fever virus in Africa. Apparently, the lesions in their Marmoset 63 were similar to those found by us in *C. albicollis*. They say in regard to their experiment: "Although there was no obvious other cause of death, the lesions in this animal did not furnish sufficient evidence for the conclusion that it had died of yellow fever infection."

Genus *Callithrix*. True Marmosets. *C. albicollis* (Spix)

Callithrix albicollis is found in great numbers in the environs of Bahia. Twenty of this species were used in the experiments here reported.

It was found comparatively easy to pass yellow fever virus through these marmosets and back to *rhesus* monkeys, either by blood transfers

Temperature Records of Monkeys Infected with Yellow Fever

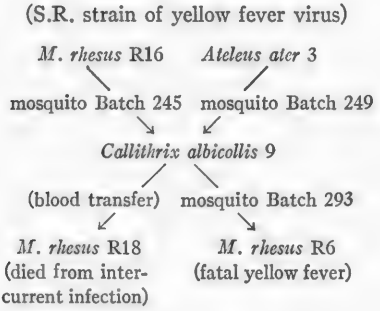
Genus and number	Date of beginning of experiment	Virus strain	Manner of infection	Transfer from:	Temperature A.M. and P.M. (degrees F.)											Outcome
					Initial day of experiment	1	2	3	4	5	6	7	8	9	10	
Callithrix 4	10/29/29	Asibi	Injection of blood	*M. rhesus* R1	— 101.7	100.5 101.2	101.9 102.1	103.5 104.0	103.8 103.7	103.5 103.8	103.6 102.0	D				Died (yellow fever?)
Callithrix 7	11/12/29	Asibi	Mosquito bites Batch 248	*M. rhesus* R15	— 101.6	101.2 101.9	101.6 102.0	102.6 102.4	102.1 101.9	101.6 101.7	101.9 102.0	101.8 101.9	102.5 102.2	102.0 102.5	101.7 101.9	Recovered
Callithrix 8	11/12/29	S.R.	Mosquito bites Batch 245	*M. rhesus* R16	— 102.7	101.9 101.6	101.2 101.9	101.7 101.5	101.8 102.2	101.9 102.9	102.4 101.9	101.7 101.6	101.2 101.0	101.7 101.9	102.9 102.4	Recovered
Callithrix 9	11/22/29	S.R.	Mosquito bites Batches 245 and 249	*M. rhesus* R16 *Ateleus* 3	— 101.7	101.9 101.5	101.7 102.0	101.9 101.7	102.0 102.8	D						Died as result of bleeding?
Callithrix 12	12/13/29	S.R.	Mosquito bites Batches 283 and 284	*M. rhesus* R17	101.6 102.2	102.5 102.4	102.7 103.5	101.9 103.1	103.0 102.5	102.4 102.9	102.7 102.5	102.9 103.0	102.7 102.9	102.5 103.0	103.7 102.9	Died as result of bleeding?
Callithrix 13	12/16/29	S.R.	Injection of blood	*Callithrix* 12	102.0 101.8	102.2 103.4	101.0 102.6	102.0 102.9	103.2 103.5	100.0 95.4						Died as result of bleeding?
Callithrix 15	12/21/29	S.R.	Injection of liver emulsion	*Callithrix* 13	— 102.0	102.4 102.1	102.5 102.7	102.6 102.4	102.5 102.5	102.4 103.0	102.9 103.2	103.7 103.6	103.4 103.5	103.6 103.4	D	Cause of death undetermined

Callithrix 17	12/26/29	Asibi	Mosquito bites Batches 275 and 276	*Callithrix* 4	—	101.6	102.0	102.2	103.0	103.5	102.7	103.4	103.0	103.5	97.9	Died on 15th day, cause unknown
				Leontocebus 1	101.9	101.8	101.9	102.5	102.9	103.7	102.9	102.6	103.5	103.2	102.0	
Callithrix 18	12/30/29	Asibi	Injection of blood	*Callithrix* 17	—	103.1	102.2	103.0	103.4	103.5	103.6	103.8	103.9	104.0	104.0	Recovered
					101.8	103.5	103.2	103.3	103.6	103.7	103.6	103.7	103.8	103.9	104.2	
Callithrix 19	1/ 3/30	Asibi	Injection of blood	*Callithrix* 18	101.6	102.0	101.2	101.9	102.2	102.4	102.0	101.7	102.5	94.0		Killed when moribund. Ulcer of stomach
					101.9	101.9	101.8	102.0	102.1	102.2	101.9	102.1	99.7	D		
Callithrix 20	1/ 8/30	Asibi	Injection of blood	*Callithrix* 19	101.9	102.2	102.0	104.0								Killed when moribund (yellow fever?)
					102.1	102.0	105.5	94.0								
Leontocebus 1	11/ 2/29	Asibi	Injection of liver emulsion	*M. rhesus* R12	—	102.2	103.6	104.0	103.7	103.6	104.2	103.8	102.9	D		Died (yellow fever?)
					101.5	103.0	104.5	103.9	103.8	104.6	104.0	103.6	102.0			
Leontocebus 2	1/29/30	Asibi	Mosquito bites Batch 324	*M. rhesus* R11	—	102.1	103.5	104.4	103.6	103.6	103.4	103.9	104.8	103.9	103.5	Died on 13th day (yellow fever?)
					101.9	102.7	103.9	103.9	103.8	103.7	103.6	104.0	104.0	103.7	102.9	
Leontocebus 3	2/ 1/30	Asibi	Injection of blood	*Leontocebus* 2	—	103.1	103.5	103.6	103.7	D						Died as result of bleeding?
					102.0	102.9	103.7	103.8	101.8							
Leontocebus 4	2/ 4/30	Asibi	Injection of blood	*Leontocebus* 3	—	102.8	104.8	104.4	104.5	104.0	104.2	103.9	103.1	102.2	102.4	Killed when moribund on 11th day (yellow fever?)
					101.9	104.6	104.7	104.1	104.2	104.5	104.0	103.6	102.7	102.1	101.9	
Leontocebus 5	2/ 5/30	Asibi	Injection of blood	*Leontocebus* 4	—	102.2	103.9	104.0	104.1	104.0	104.0	103.9	104.2	103.9	D	Died (yellow fever?)
					100.9	103.0	104.2	103.9	104.0	104.2	104.1	104.0	104.1	102.2		

or by mosquito feeding. However, after the virus was carried through a series of marmosets, it appeared to decrease in virulence for *M. rhesus*. This was not the case when the virus was passed through certain other species of Brazilian monkeys. A very few of the marmosets showed a definite fever (104°F., or above). Most of them died while under observation, but the lesions found at necropsy did not resemble those of yellow fever as seen in humans, in the *rhesus* monkey, and in certain other species of monkeys.

Experiment I.—On October 29, 1929, Marmoset 4 was inoculated intraperitoneally with 2.5 cc. of citrated blood, containing Asibi strain virus, from *M. rhesus*

Scheme I



R1. The animal's initial temperature was 101.7°. On November 1 the temperature reached 104°, blood was transferred to *M. rhesus* R2, and mosquito Batch 275 was allowed to engorge. Marmoset 4 was found dead on November 4. There was a little dried blood about the nose and mouth. Jaundice was doubtful. The lungs showed a patchy congestion with, perhaps, a trace of bronchopneumonia. The liver had a slate-colored surface, but on section appeared yellow and unmistakably fatty; the organ was friable. The spleen was enlarged; its pulp was soft and its follicles were small. Kidney sections were opaque, with slight bile-staining and injection of the vessels. The gastro-intestinal tract was negative. Microscopically the liver showed degeneration, mainly midzonal, including a few necrotic cells. Fat was present in large amount, with the heaviest deposition in the midzones. A few leucocytes were noted. *M. rhesus* R2 died on the fifth day with yellow fever. Mosquito Batch 275 fed on *M. rhesus* R3 on November 21. This animal also died on the fifth day with typical yellow fever.

Experiment II.—On November 12, 1929, Marmoset 7 was fed upon by mosquito Batch 248, infected with Asibi strain virus. The initial temperature was 101.6°.

There was no subsequent fever and no significant rise in temperature (see table). However, on November 16, 1.5 cc. of blood was taken from the heart and injected into *M. rhesus* R4, which died on the sixth day with typical yellow fever.

Experiment III.—Marmoset 8 was fed upon November 12 by mosquito Batch 245, infected with the S.R. strain of virus. On the fourth day (November 16) blood was withdrawn for injection into *M. rhesus* R5. This monkey died on the fifth day with typical yellow fever. Marmoset 8 showed no significant rise in temperature at any time.

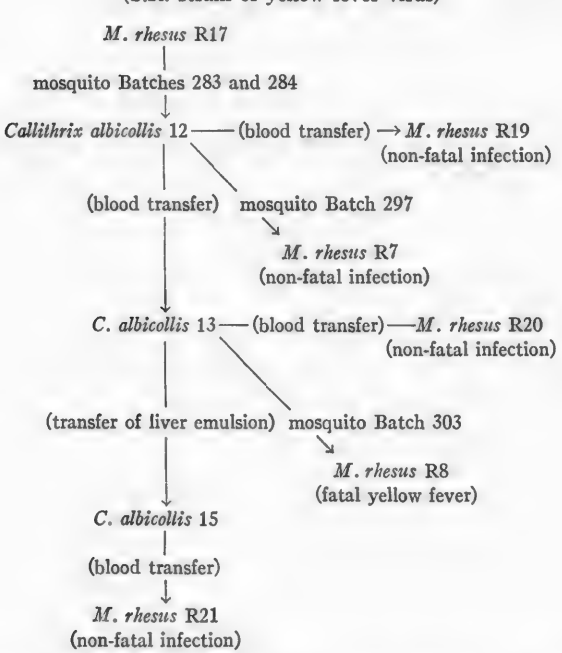
Experiment IV.—(Scheme I.) On November 22, 1929, Marmoset 9 was fed upon by mosquito Batches 245 and 249, infected with the S.R. strain of virus. The initial temperature was 101.7°. On November 26 the temperature in the afternoon was 102.8°. The animal was bled, and mosquito Batch 293 was allowed to engorge. On the following morning more blood was taken, and the monkey died soon thereafter. At necropsy the centers of the liver lobules showed as red dots surrounded by zones of an orange-yellow color; the organ was friable. The gastric mucosa showed a few dark streaks which appeared to be changed blood. The other organs revealed nothing of note. Microscopically, the lobules of the liver were seen to have a certain amount of midzonal degeneration, with deposition of fat, and perhaps a few necrotic cells. There was congestion, and many leucocytes were observed (mainly polymorphonuclear, occurring in small nests). The kidneys showed congestion and a slight cloudy swelling. On December 16 mosquito Batch 293 fed on *M. rhesus* R6, which died on the fifth day with typical yellow fever.

Experiment V.—(Scheme II.) This experiment consisted in the infection of Marmoset 12 by mosquitoes carrying the S.R. strain of virus, passage of the virus to Marmoset 13 by the injection of blood taken from Marmoset 12, and to Marmoset 15 by injection of emulsion of liver from Marmoset 13. Blood transfers to *rhesus* monkeys from Marmosets 12, 13, and 15 all produced definite infection, with fever and subsequent immunity to reinoculation, but no deaths. Mosquito transfer from Marmoset 12 to *M. rhesus* R7 was also non-fatal. However, mosquito Batch 303, infected on Marmoset 13, induced by its bites a fatal infection in *M. rhesus* R8. None of the marmosets had a definite fever (see table), and at necropsy none of them showed lesions suggestive of yellow fever.

Experiment VI.—(Scheme III.) In this experiment virus was passed to Marmoset 17 by mosquitoes, themselves infected on marmosets, and from Marmoset 17 through Marmosets 18 and 19 to Marmoset 20 by direct transfer of blood, taken in each case on the fourth day following inoculation. Blood from Marmoset 17 and the bites of mosquitoes fed on this animal caused fatal infections in *rhesus* monkeys. Blood virus from Marmoset 18 produced a fatal infection in *M. rhesus* R9. Blood from Marmosets 19 and 20 caused non-fatal infections in *M. rhesus* 10 and 11, and the bites of mosquito Batch 326, fed on Marmoset 20, produced non-fatal infections in two *M. rhesus*. Apparently the virus became attenuated for the *rhesus* monkeys by repeated passage through the marmosets. The last animal

in the series, Marmoset 20, had a clinical course suggestive of yellow fever. The initial temperature on January 8, 1930, was about 102°. On January 10 the temperature suddenly rose to 105.5°, at which time mosquitoes were fed and transfer of blood was made. On the afternoon of January 11 the temperature dropped to 94°, and the animal was killed. At necropsy there was a suggestion of icterus in the tarsal plates. The liver was a pale yellow; a small roundworm was found coiled up in one lobe. The other organs were essentially negative.

Scheme II



Microscopical examination showed the liver to be nearly one half necrotic. The necrosis was primarily central, but extended into the midzones. There was a heavy leucocytic invasion. The kidneys showed a very slight cloudy swelling.

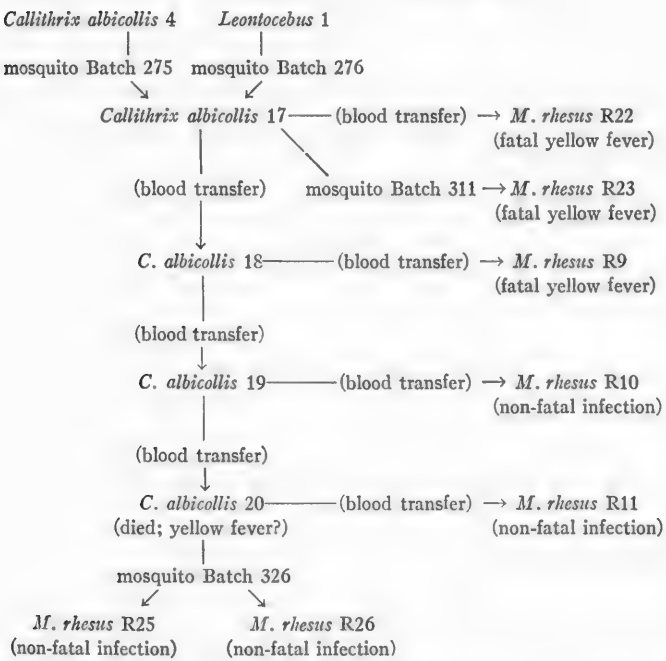
Genus *Leontocebus*. Tamarins. *Leontocebus ursulus* (Humboldt)

The little black tamarin, or marmoset *Leontocebus ursulus*, comes from Para. Elliot (5) calls it *Cercopithecus ursulus*. We were able

to secure five specimens for experimental work. These animals proved to be more susceptible to yellow fever virus than *C. albicollis*. Four of the five developed fever; all five died, two of them showing marked liver necrosis (Fig. 1), and two others the most intense jaundice that we have ever seen in monkeys.

Scheme III

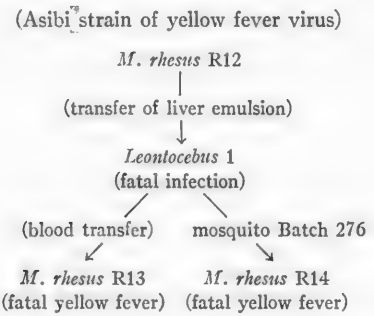
(Asibi strain of yellow fever virus)



Experiment VII.—(Scheme IV.) On November 2, 1929, *L. ursulus* 1 was inoculated subcutaneously with 1.5 cc. of liver emulsion, containing Asibi strain virus, from *M. rhesus* R12. The initial temperature was 101.5.° On November 4 the temperature rose to 104.5°, at which time blood was transferred to *M. rhesus* R13 and mosquito Batch 276 was allowed to engorge. *M. rhesus* R13 died on the sixth day with typical yellow fever. Mosquito Batch 276 later induced a fatal attack of yellow fever in *M. rhesus* R14. *L. ursulus* 1 was found dead on No-

vember 11. Icterus was marked in the tarsal plates and in the intima of the aorta. A small abscess was found in the anterior abdominal wall at the point of injection of liver emulsion. The liver was rather friable, with sharp edges; the peritoneal surface was reddish brown, with purple discolorations in places. The cut surface was also reddish brown, with indistinct lobular markings. The spleen was swollen, with rounded edges, and was congested and purplish in color. The other organs did not appear abnormal in the gross. Microscopically, the liver showed a severe, but irregular, necrosis. In the midzones frequent islands and strands of cells were left intact. Necrosis sometimes extended to the central veins. Near the capsule several areas were noted where necrosis was complete and digestion advanced.

Scheme IV

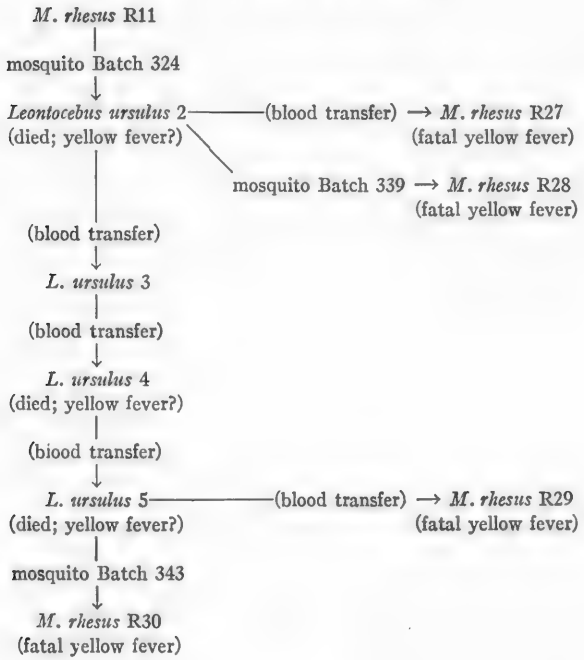


Experiment VIII.—(Scheme V.) On January 29, 1930, mosquito Batch 324, infected with Asibi strain virus, fed upon *L. ursulus* 2. The strain was passed in succession through *L. ursulus* 2, 3, 4, and 5 and back to *M. rhesus* from the last marmoset by blood transfer and by mosquito bites, producing fatal infections. Transfers from *L. ursulus* 2 also induced fatal infections in *rhesus* monkeys; no transmission to *M. rhesus* was attempted from *L. ursulus* 3 and 4. The temperature reactions are given in the table. Although intense icterus was noted at autopsy in *L. ursulus* 4 and 5, the most remarkable lesions were found in *L. ursulus* 2. This animal had fever on February 1 and again on February 5 and 6. It died on February 11. Marked icterus was noted in the tarsal plates, in the intima of the aorta, and even in the skin. The liver was friable and of a curiously mottled yellow, orange, and brown (in general, lighter than normal). The spleen appeared to be swollen. The kidneys were pale and bile-stained. The stomach contained food mixed with a considerable amount of changed blood. Microscopically, the liver showed over 80 per cent necrosis, essentially midzonal, with rings of cells, relatively unaffected (except for fat), around the vessels. Occasional nests of intact cells were also found in the midzones. The necrotic cells showed very

eosinophilic cytoplasm and nuclei in various stages of degeneration and fragmentation. There was congestion of vessels, generalized pigmentation, and deposition of fat (especially in cells around the central veins). Round-cell infiltration and hyperplasia of epithelium of the bile-ducts was noted in the portal spaces. The kidneys showed slight cloudy swelling and injection of the glomeruli.

Scheme V

(Asibi strain of yellow fever virus)



DISCUSSION

The experiments here presented show that yellow fever virus can be introduced into the marmosets, *Callithrix albicollis* and *Leontocebus ursulus*, by mosquito bites, that it can be passed through at least four animals and can be taken out again by mosquitoes which subsequently are able to transmit the infection to *rhesus* monkeys. There is some evidence that in such a series of passages through *C. albicollis*

the virus becomes somewhat attenuated for *M. rhesus*. Apparently no change in the virus takes place in passage through *L. ursulus*.

From five *C. albicollis*, numbers 3, 5, 7, 8, and 18, convalescent serum was used in protection tests against Asibi strain virus. Protection was secured in every instance with these convalescent sera, but not with serum from a normal marmoset. Numbers 7, 8, and 18 are mentioned in the protocols. Numbers 3 and 5 were inoculated with blood containing virus but neither showed a febrile reaction; no attempt was made to transfer the virus from them to other monkeys.

The lesions found at necropsy in *C. albicollis* did not closely resemble those found in human beings or *rhesus* monkeys that had died of yellow fever. On the other hand, the liver lesions in two of the five *L. ursulus* were very extensive and comparable to the usual midzonal necrosis of susceptible species.

The fact that the virus of yellow fever can be passed through marmosets with comparative ease may have some importance in the epidemiology of the disease in nature. These little monkeys are frequently kept as household pets, and even the wild ones are often found on uncleared land within urban limits. It is conceivable that they might pick up yellow fever from domestic sources, carry it to outlying districts, and aid in its wide dissemination. Such a course of events seems the more possible since we know that various "wild" species of mosquitoes are fairly efficient vectors of the virus.

SUMMARY AND CONCLUSIONS

1. It has been possible to introduce yellow fever virus into the small Brazilian monkeys, *Callithrix albicollis* and *Leontocebus ursulus*, by the bites of infected mosquitoes and to carry the virus through a series of four passages in each species and back to *rhesus* monkeys by the bites of *Stegomyia* mosquitoes fed on the last marmoset of each series.

2. Five specimens of *L. ursulus* were used. Four developed fever, and all died during the experiments. At least two showed liver necroses comparable to those found in human beings and *rhesus* monkeys that died of yellow fever.

3. Twenty specimens of *C. albicollis* were used. Very few showed a

temperature reaction following the introduction of virus. Of those that died, none had lesions typical of yellow fever as seen in certain other species of monkeys and in humans.

4. The convalescent serum from each of five *C. albicollis* protected a *rhesus* monkey against yellow fever virus, but the serum from a normal marmoset of the same species was found to be non-protective.

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3. Davis, N. C., *Jour. Exp. Med.*, 1930, **51**, 703.
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EXPLANATION OF PLATE 16

FIG. 1. Section of the liver of a black marmoset which died of yellow fever. The tissue shows marked necrosis. (× 320.)

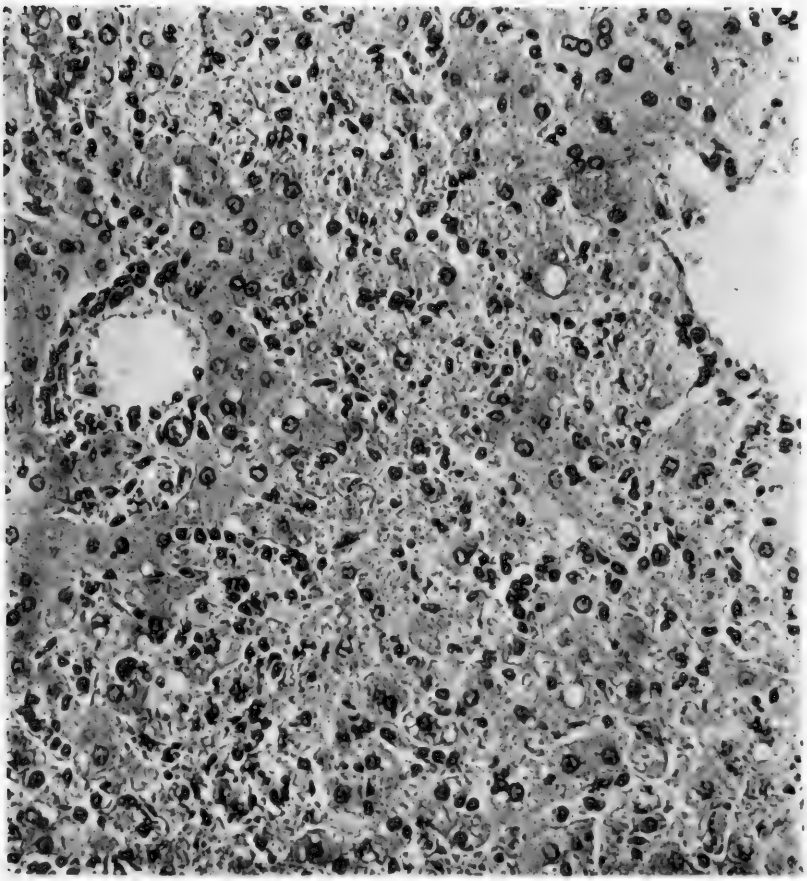

FIG. 1

(Davis: Susceptibility to yellow fever virus)

UNIV. OF MICH.

THE EXTRACTION OF THE CELL CONTENT OF MICRO-ORGANISMS BY PERCUSSION OF THE FROZEN CELLS

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INTRODUCTION

For many purposes it is desirable to obtain extracts of microorganisms without delay and free from the changes which undoubtedly accompany the procedures now used. The present methods involve chemical agents, autolysis, prolonged alternate freezing and thawing, or dessication and grinding. These processes often bring about changes which may effect the nature of the extract. The method of the Buchner press, which has been used for obtaining yeast extracts, while avoiding some of the objectionable features of other procedures is apparently not applicable for general use with pathogenic bacteria. It occurred to us that if microorganisms were frozen very quickly so that the ice crystals were very fine, and if this mass of cells containing the fine crystals were then subjected to sudden high pressure, such as may be obtained by blows applied to a piston in a closed cylinder, the friction of the ice crystals might either disrupt the cells or so change the character of the membrane that the intra-cellular material would be forced out. The apparatus described here was designed to test this idea, and as will be seen, its use has given encouraging results.

Apparatus and Materials

The apparatus employed consisted of a cylindrical steel chamber and a closely fitting steel plunger so cut as to move freely without compression. The chamber was made by cutting a hole, 1.6 cm. in diameter and 8.5 cm. deep in a piece of steel shafting 10 cm. in diameter and 12.5 cm. long, with a machine cutting tool. The chamber was flat at the bottom. About 1 cm. from the top of this chamber a cir-

cular groove was cut to form an overflow chamber having a capacity of about 6 cm. The plunger was made of high grade steel tempered at the lower end.

The cells which were used for extraction were pure cultures of bakers' yeast, 24-hour broth cultures of *B. coli*, *B. xerosis*, *Streptococcus hemolyticus*, Pneumococcus Type II, a 5-hour dextrose broth culture of Pneumococcus Type I, 4-day cultures of *B. dyphtheriae* and a 5-day glycerine bouillon culture of the bovine tubercle bacillus. The cells were repeatedly washed with distilled water until the supernatant obtained by centrifuging no longer gave the biuret reaction for proteins and were then suspended in distilled water.

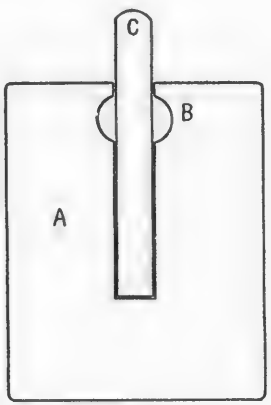

FIG. 1. Extraction apparatus consisting of a steel chamber, A, having a reservoir, B, and fitted with a tempered steel plunger, C.

EXPERIMENTAL

Before each experiment the apparatus was cleaned by scouring and washing and sterilized in a hot air oven. It was then cooled, first by packing with a salt and ice mixture and then by placing into an insulated container and packing with solid CO_2 (dry ice). The chamber was then charged with 2 cc. of the suspension of cells and the plunger replaced and fastened with a rubber collar so as to remain suspended about 1 cm. above the charge. The top of the apparatus was then covered with sterile cotton. After 20 minutes additional cooling in dry ice, the apparatus, along with its insulated container, was placed on a heavy metal base and the plunger struck twenty-five heavy blows with an 8-pound hammer. This subjected the cells to the crushing action of the ice crystals. Intervals of 10-seconds or longer between blows were allowed to prevent the charge from being forced into the overflow chamber by the melting effect of pressure and heat before the desired number

of blows had been struck. The charge was thawed, by placing the apparatus in a water bath, and was then removed with a pipette. After centrifuging, the supernatant liquor was passed through a Berkefeld filter.

All of the supernatants and filtrates obtained by subjecting the various microorganisms to this treatment were slightly opalescent, frothed readily and gave a heavy precipitate when a few drops of 10 per cent acetic acid were added. They always gave the biuret reaction for proteins but generally only a weak Molisch test for carbohydrates. All of them became more opalescent when boiled and in some cases a slight precipitate was obtained. For control in each case a portion of the same suspensions of the microorganisms used in the experiments was frozen with dry ice, thawed, and the supernatants, obtained by centrifuging, tested with the biuret and acetic acid precipitation tests. In every instance these tests on the controls gave negative results.

Morphologic evidence of cell destruction was obtained in case of yeast cells and pneumococci. Practically all of the yeast cells were found to be without vacuoles after percussion. There was also some evidence of cell fragmentation. Pneumococcus cells which before treatment were Gram positive, after treatment showed many Gram negative cells with débris. After a second treatment of these same cells only occasional whole cells were visible. The preparation showed a large amount of Gram negative cell detritus.

Gram stain preparations of *B. xerosis*, *B. dyphtheriae* and hemolytic streptococcus gave unconvincing evidence of any change in staining reaction or morphology. The acid fast character of the bacillus of bovine tuberculosis was not found to be changed.

DISCUSSION

The microorganisms used in this investigation were chosen with respect to their varied resistance to other methods of extraction. The uniformly positive results which were obtained indicate that this method should have a wide range of application in obtaining similar extracts from other species of resistant organisms. The treatment of frozen cells by percussion should facilitate further extraction of cell residues by chemical means.

The chief advantages of this method, however, lie in the possibility

of obtaining for immunological and biochemical studies fresh extracts of living cells, which have been kept at low temperature, thus minimizing the changes brought about by autolysis, and which are free from chemical contamination.

SUMMARY

A method is described for rapidly obtaining fresh extracts of microorganisms by percussion of the frozen cells.

Filtrates giving the biuret reaction and yielding heavy precipitates upon the addition of acetic acid were obtained from the washed cells of cultures of yeast, hemolytic streptococcus, pneumococcus, *B. coli*, *B. xerosis*, *B. dyphtheriae* and the bacillus of bovine tuberculosis.

THE RELATION OF NATURAL HUMORAL ANTIPNEUMOCOCCAL IMMUNITY TO THE INCEPTION OF LOBAR PNEUMONIA

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Further progress in our understanding of lobar pneumonia in man not improbably depends to a great extent upon the determination of those conditions necessary for the inception of the disease, concerning which information is at present scarcely more than speculative. While the implantation of pathogenic pneumococci* by inhalation is presumably essential for the production of the pneumonic lesion, it does not seem likely that this is usually the deciding factor, for the reason chiefly that contact infection occurs so infrequently. Protection from infection may be due in part to the barrier interposed by the devious channels of the upper respiratory tract to the passage of the pneumococcus-containing droplets, but when the concentration and fineness of the droplets is sufficient, some of them reach the lung, as can be shown experimentally. Disturbed function of the respiratory tract accompanying mild infection of the upper or lower respiratory passages or following operations, especially on the upper abdomen, precedes the onset in a considerable percentage of pneumonia cases. In others with an antecedent chilling a general alteration in susceptibility may possibly take place as well. However, for those numerous instances in which pneumonia occurs suddenly during a period of apparent normal health another explanation must be sought.

Blake and Cecil's work on the experimental production of the disease in monkeys (1) suggests that in order to initiate lobar pneumonia it is

* Since pneumococci even of low virulence are capable of causing pneumonia under certain conditions provided they are "S" producing, it seems proper to term all such pneumococci as pathogenic.

only necessary to implant in the lungs a sufficient number of pneumococci of adequate virulence for the animal. In their experiments the number of organisms employed was very small. But other authors (2), working with dogs which possess much higher natural antipneumococcus immunity than the species of monkeys used above, have found that the simple intrapulmonary implantation of pneumococci either failed to cause pneumonia or produced the disease only if used in quantities enormously greater than could possibly occur in the natural acquisition of the disease in man. Is the difference in the ease with which lobar pneumonia can be induced in these two animal species due simply to their relative susceptibility to pneumococcus infection?

A previous study on the resistance of normal human beings to recently isolated strains of pathogenic pneumococci (3) has indicated that as a group they possess considerable natural immunity to these organisms as shown by the pneumococcus-destroying power of their blood which in degree is not much below that of the dog. But individuals were found to vary greatly in their reaction toward the several types of pneumococci. This ranged from marked killing power of the blood for one type to none against others. The fluctuations in antipneumococcus action were found to reside in the serum, not in differences in the leucocytes. These findings indicated the feasibility of securing further information about one of the factors which is possibly concerned in the inception of lobar pneumonia, namely the amount of circulating specific antipneumococcus substances at the onset of the disease.

Methods

The pneumococcidal tests with human serum and leucocytes were carried out as previously described (4, 5). Serum samples were secured from the patient on admission and daily thereafter throughout the course of the disease. These were kept in an atmosphere of CO_2 at 4°C. and all were tested at one time.* Frequently in early cases the serum was tested as soon as the homologous pneumococcus was isolated. In every instance the experiments were performed with the organism isolated from the patient's lung, blood or sputum, and a control consisting of the pooled serum of four to seven normal individuals was run at the same time. The smallest amount of pneumococcus suspension used was 10^{-7} in all but cases V. C.

* Serum preserved in this way showed no deterioration in its pneumococcidal-promoting properties for a period of 6 to 7 days.

and H. F. P. in which 10^{-8} was employed. Quantities less than 10^{-7} not infrequently led to irregularities in the test. In a number of cases the opsonic and mouse protective properties of the serum were determined also. In almost all instances the tests were repeated in order to verify the results. Blood cultures were taken in the usual manner, 6 to 10 cc. into broth and 1 cc. and 2 cc. into plates.

Clinical Cases

The present study comprises twelve cases of lobar pneumonia in whom observations were begun from 4 to 48 hours after the onset of the disease. The antipneumococcus properties exhibited by the serum within this period may be considered with a fair degree of certainty as referable to the class of natural immune substances, since acquired immune bodies have not been observed by us to develop before the third day of the disease.* The results of the tests as summarized in Table I revealed the unexpected finding that in the majority of cases the initial serum specimens possessed pneumococcidal-promoting properties, sometimes slight and again as great as in normal individuals.** In only four cases was this property absent. The possibility that an organism other than that causing the disease was responsible for the serum differences, and in particular the exhibition of well marked antipneumococcus properties, is excluded by the fact that pneumococci isolated from the lung and blood stream in certain cases were affected to as pronounced a degree as were the organisms obtained from the sputum in others. Nor did the virulence of the pneumococcus strains appear to bear any relationship to the action on them of the patient's serum. Organisms against which one patient's serum-leucocyte mixtures showed marked killing action were fully as virulent when tested by animal inoculation and growth capacity in normal pooled human serum and leucocytes, as were those on which another patient's serum had no effect. That is, the variations appeared to reside in the individual rather than in the several pneumococcus strains. Again, the initial pneumococcidal-promoting properties of the serum could not be associated with the extent of the lung lesion, as determined by

* As will be shown in a succeeding communication, acquired humoral immunity does not usually occur before the fourth or fifth day of the disease.

** By normal degree is meant the pneumococcidal-promoting action of pooled normal human serum for the particular pneumococcus in question.

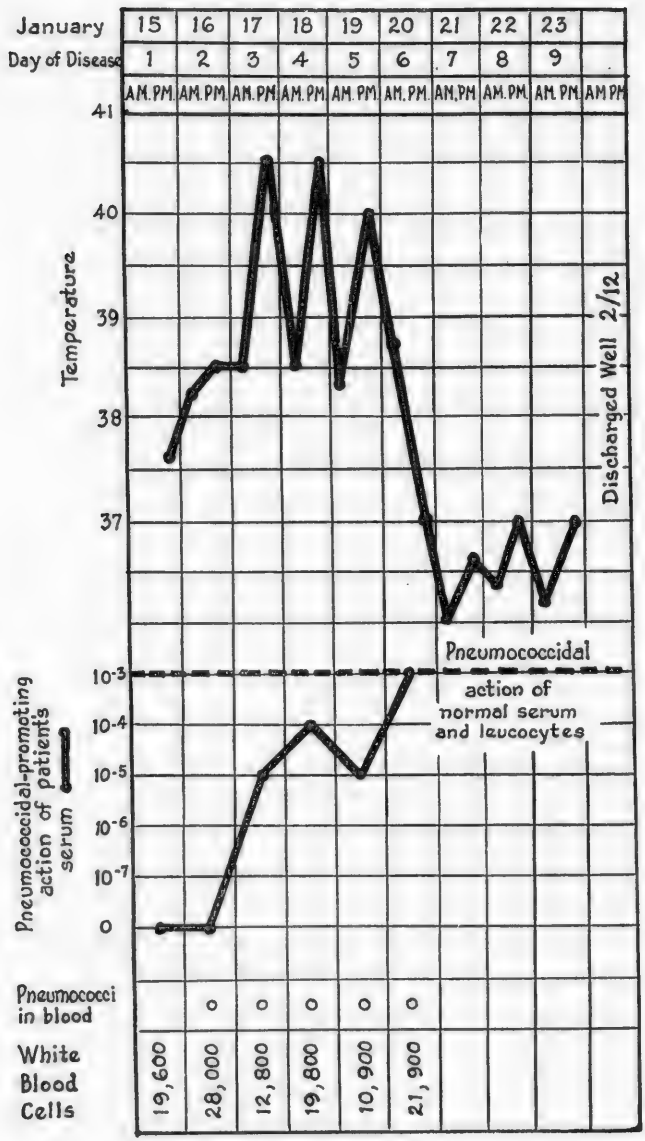
TABLE I

Case	Type	Pneumococcus isolated from	Pneumococcidal action of serum and blood invasion	Time from onset within which blood specimens were secured								Outcome
				24 h.	48 h.	72 h.	4 da.	5 da.	6 da.	7 da.	8 da.	
R. E.	II	Sputum	No. Pn. killed	10^{-3} (4 h.)	10^{-3}							Rec'd within 18 hrs.
			Pn. in blood	0	—							
A. L.	I	Sputum	No. Pn. killed	0 (6 h.)	0	10^{-5}	10^{-4}	10^{-5}	10^{-3}			Rec'd 6th day
			Pn. in blood		0	0	0	0	0			
A. M.	II	Sputum	No. Pn. killed	10^{-6} (9 h.)	(Serum treated)							Rec'd 6th to 7th day
			Pn. in blood	0	—	0	0					
R. M. E.	IIa	Lung	No. Pn. killed	10^{-5} (18 h.)	10^{-7}	(Serum treated)						Rec'd 7th day
			Pn. in blood	0	0	0						
J. T. A.	I	Sputum	No. Pn. killed	0 (22 h.)	(Serum treated)							Rec'd 5th day
			Pn. in blood	0	0	0						
K. H.	III	Sputum	No. Pn. killed	10^{-5} (22 h.)	10^{-5}	10^{-5}	10^{-4}	10^{-4}	10^{-4}	10^{-4}		Rec'd 7th day
			Pn. in blood	0	0	0	0	0				

E. J.	IV	Sputum	No. Pn. killed	10^{-6}	10^{-6}	10^{-5}	10^{-5}	10^{-4}				Rec'd 5th day
			Pn. in blood	−	−	−	−					
H. C.	III	Blood	No. Pn. killed	10^{-7}	10^{-7}	10^{-6}	10^{-5}					Rec'd 4th day
			Pn. in blood	+ (1 col.)	+ (1 col.)	0	0					
J. V.	II	Blood	No. Pn. killed		0							Died in 48 hrs.
			Pn. in blood		+							
F.	IIa	Sputum	No. Pn. killed		10^{-4}	10^{-5}	10^{-5}	10^{-5}	10^{-4}	10^{-4}		Rec'd 7th to 8th day
			Pn. in blood		0	0	0	0	0	0		
H. F. P.	IV	Lung	No. Pn. killed		10^{-5}	10^{-6}	10^{-7}	10^{-8}	10^{-8}	10^{-7}	10^{-8}	Died 8th day
			Pn. in blood		0	0	+ 1 col.	+ 12 col.	+ 4 col.	+ 39 col.	+ 56 col.	
V. C.	III	Blood	No. Pn. killed		10^{-4}	0	0					Died 6th day
			Pn. in blood		0	+	+ 12 col.	+	+ 86 col.			

The minus powers of 10 above, represent the amounts of the standard pneumococcus containing 1 billion pneumococci per cubic centimeter which were killed by the serum and leucocytes obtained from approximately 0.5 cc. of blood.

− = Not done.



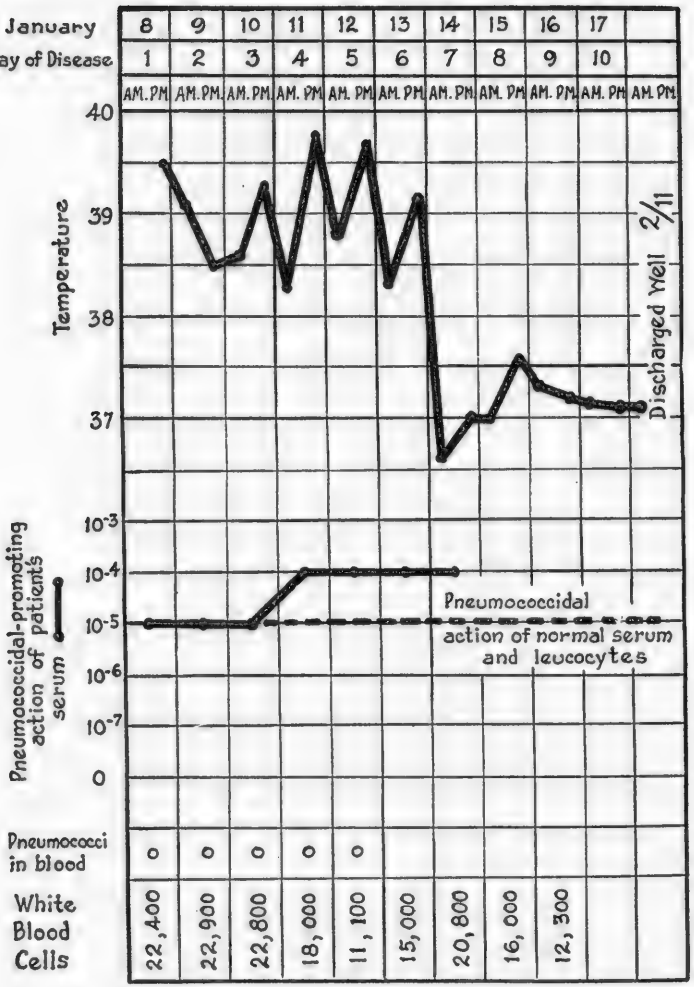
TEXT-FIG. 1. Case A. L., lobar pneumonia—Pneumococcus Type I.

X-ray and physical signs. Both cases A. L. and J. T. A., in whom a very small lesion existed at the time of the first observations, showed no pneumococcus immune properties in the blood serum. On the other hand, patients K. H., V. C. and H. F. P. showed lesions of considerable extent and a normal or at most a slight reduction in degree of pneumococcus-killing power in their blood. The number of cases is far too limited to permit of any conclusions in relation to pneumococcus type.

With the progress of the disease, changes in the pneumococcidal-promoting action of the serum usually followed one of several fairly regular courses. When antipneumococcus properties were not demonstrable at the beginning, none appeared until the onset of acquired immune-body production. Case A. L., Text-fig. 1, is an example. There were a number of other patients concerning whom data are not given in these tables (since observations were not begun until after 48 hours) whose blood serum exhibited the same findings.* In contrast were patients such as case K. H. (Text-fig. 2), who showed a well marked degree of pneumococcidal-promoting serum power all through the disease. A third course is shown by case V. C., Text-fig. 3. Following an initial normal titer of antipneumococcus properties there occurred a progressive and sometimes very rapid disappearance of the serum effect which did not reappear unless recovery took place. Of course all the patients studied did not fit exactly into these three catagories, case H. C., for example, being an exception but they could practically all be classified as modification of one of them.

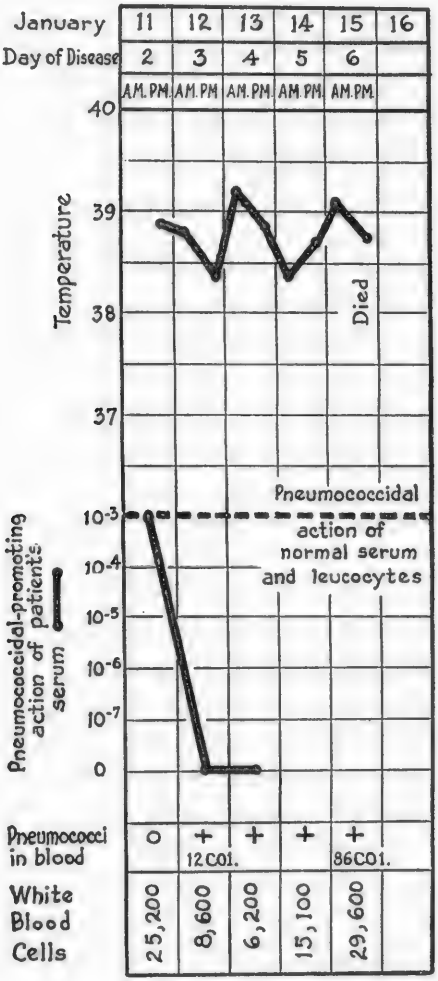
A constant relationship was found to exist between the concentration of immune properties in the serum and blood invasion. In the presence of a killing power of the serum and leucocytes represented by the destruction of as small an amount as 10^{-6} of the standard pneumococcus suspension, pneumococci were not found in the blood stream. When pneumococcidal action fell below this point blood invasion tended to occur. That it did not always occur even when antipneumococcus properties were not demonstrable in the serum may be taken as an indication that a modicum of this action still persisted. Indeed when the tests were carried out with numbers of pneumococci as few as 10^{-8} of the suspension (containing actually less than 10 pneumococci)

* The details of the studies on these patients will be given in a following communication.



TEXT-FIG. 2. Case K. H., lobar pneumonia—Pneumococcus Type III.

a residuum of pneumococcus-killing power was sometimes found to persist even in the presence of an increasing blood invasion, as in the instance of case H. F. P. Probably other factors participate in con-



TEXT-FIG. 3. Case V. C., lobar pneumonia—Pneumococcus Type III.

trolling the penetration of pneumococci into the blood channels, such as the degree and perhaps nature of the cellular reaction in and surrounding the pneumonic lesion, but the pneumococcidal action of the blood would seem to be the one of chief importance.

Because of the observations of other workers who have studied the immune properties of the serum during lobar pneumonia by means of mouse protection, a word should be said concerning the relative sensitiveness of the pneumococcidal reaction as carried out in the present investigation and the mouse test. A comparison of these two techniques on the same serum specimens has shown the pneumococcidal test to be the more sensitive. A serum which causes killing of pneumococcus in the serum-leucocyte mixture in quantities as large as 10^{-5} and 10^{-6} of the standard suspension will often fail to cause protection of mice against amounts greater than 10^{-7} of the same suspension or exert no protection at all. Hence it is not surprising that most workers have failed to find mouse protective properties in the serum during any but the later stages of the disease or have found them only to a degree that seemed negligible on account of the individual variations exhibited by mice.

DISCUSSION

The findings at once suggest the question, does the presence of pneumococcidal properties in the blood after the onset of the disease indicate that they were there just prior to or at the inception of the pneumonic process. While no direct answer can be given, observations on the experimental disease in animals and on human beings with pneumonia afford data for certain assumptions in the affirmative. Marked and rapid fluctuation in the degree of natural pneumococcidal activity of the blood has not been noted in either man or animals during health or in disease. In the experimental animal, as one of us has shown (6), a generalized pneumococcus infection is accompanied by a pronounced and progressive diminution in circulating antipneumococcus immunity which does not reappear until the development of acquired immune substances occurs coincidentally with recovery. However, when the pneumonia process is localized as in experimental lobar pneumonia, the natural humoral immune bodies tend to persist with little diminution throughout the course of the disease. Blood

samples taken immediately before infection is initiated show a normal pneumococcidal action. Tests on pneumonic patients indicate that once a marked decrease or disappearance of the antipneumococcus properties of the blood has occurred they do not show an increase again until shortly before or at the time of recovery.

Other workers have also observed evidence of natural antipneumococcal immunity early in the course of lobar pneumonia. Park and Cooper (7) found in more than a third of pneumonia patients they studied appreciable mouse protective action of the serum on the second day of the disease. In a paper just published Ward (8) states briefly that he found the defibrinated blood of pneumonic patients early in the disease to possess a normal pneumococcidal action against the causative organism. Details of the cases and tests are not given.

If pneumonia can develop in the presence of a normal circulating antipneumococcus defense mechanism, as seems evident from the above observations, what is the actual mechanism of infection? The most reasonable explanation would seem to be that local changes take place of such nature as to provide conditions for the growth of the pneumococci while protecting them from the pneumococcidal action of the blood. There might conceivably be failure of the normal eliminatory mechanism, tissue injury by concurrent infection, allergic reactions, or a combination of these factors. Once pneumococcus growth is established, and perhaps this may be entirely within the lumen of the bronchus—the constant diffusion of bacterial metabolic products in a circumscribed area would produce increasing tissue injury, one of the results of which has been shown to be deposition of fibrin in the capillaries and lymphatics. This would tend further to protect the pneumococci from the full action of the circulatory immune bodies. How early and to what extent the impairment of the circulation occurs in lobar pneumonia in man is not definitely known. The experimental observation of Kline and Winternitz (9), however, would suggest that it takes place early in the disease course and becomes pronounced as the lesion progresses.

The hypothesis has recently been put forward by Coryllos and Birnbaum (10) that lobar pneumonia is initiated as a result of extensive atelectasis produced by occlusion of a large bronchus. In order that the condition may occur the mucous plug must exclude

completely the passage of air and persist for some time. Pneumococci distal to the plug are thus protected from elimination. It is not our desire to enter upon a detailed discussion of this view,* but it should be pointed out that certain of the assumptions upon which the hypothesis rests are not generally supported by clinical evidence. According to Coryllos and Birnbaum, the early signs of the pneumonic lesion are found at the periphery where atelectasis occurs first, the exudative process extending progressively from the hilum to the periphery. As a matter of fact, X-ray studies in the early part of the disease show, in a large percentage of cases of lobar pneumonia, a shadow confined to the hilum which gradually spreads out to the periphery as the disease evolves. Furthermore, in none of our early cases have we found evidence of atelectasis on the affected side either by physical signs or X-ray. However, aside from the question of atelectasis and complete plugging of a bronchus, a local failure of the normal eliminatory mechanism (implied in the hypothesis of Coryllos and Birnbaum) which leads to stasis of bronchial secretion might provide, as suggested above, the environmental state necessary for effective action of the implanted pneumococci.

The rôle played by the natural circulatory immune bodies in the process of localization would seem to be to destroy pneumococci at the periphery of the lesion, thus retarding the spread of the process and limiting or preventing blood invasion. With an initial marked reduction or absence of these humoral immune factors in the presence of an infecting organism of high virulence a rapid spread of the pneumonic process and a generalized infection might be expected to occur. Whether patients such as case J. V., who died within 48 hours of an overwhelming infection were deficient in natural antipneumococcus substances at the inception of infection, can only be surmised until further data are available, but the sequence of events would suggest that such was the case.

SUMMARY AND CONCLUSIONS

A study of the pneumococcidal-promoting action of the serum of lobar pneumonia patients, secured from 4 to 48 hours after the onset of

* The subject will be considered fully in a subsequent communication on the production of experimental lobar pneumonia.

the disease, has revealed the fact that in the majority of instances the serum possessed the power to promote killing of the homologous pneumococcus, isolated in different instances from the lung, blood, and sputum. While in some instances this action was slight, in others it was present to as great a degree as in normal individuals and persisted as long as 48 hours or more after the beginning of the disease. The variations observed from case to case were not related to the extent of the pneumonic lesion or to the virulence of the several pneumococcus strains but appeared to depend on differences in individual human beings in respect to the natural antipneumococcus properties of their blood and their reaction to the invading microorganism. A constant relationship was found to exist between the concentration of immune properties in the serum and blood invasion. In the presence of a well marked pneumococcidal-promoting power pneumococci were not found in the blood stream, and only when this property was greatly diminished or lost did blood invasion occur.

The findings which are supported by certain previous experimental observations, indicate that lobar pneumonia can occur in the presence of a normal circulating antipneumococcus defense mechanism. From this it is inferred that before pneumococcus growth can be initiated there must be present in the lung local changes of such nature as to provide conditions for the multiplication of pneumococci protected from the pneumococcidal action of the blood. Suppositions as to the nature of these changes and the establishment of the pneumonic lesion are discussed.

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THE KILLING OF CERTAIN BACTERIA BY X-RAYS

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Plate 17

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The experiments to be described are an extension to soft X-rays of those[1] previously made with cathode rays. They consist essentially of studies of the time rate of killing of single bacteria by the general radiation from a tungsten tube operated at low voltage and by the characteristic K-radiation of copper.

EXPERIMENTAL

The organisms *B. coli* and *B. aertryke* were chosen for these observations because they readily give the distribution of single cells that is needed if statistical counts are to have meaning. The bacteriological procedures were identical with those already described. As before, standard cultures were provided by Dr. L. T. Webster of this Institute. Spreads of single bacteria upon the surface of agar (ca 200 organisms per in.^{2}) were irradiated before multiplication could take place. After incubation, counts were made of the number of colonies growing out upon a stamped irradiated area and upon an equal and adjacent control area marked at the time of irradiation. From these data survival ratios have been calculated which, together with measurements of the X-ray intensity striking the agar plate, can be made to give information concerning details of the killing action of the rays. In order that the survival ratios obtained in this way should refer to enough organisms to be statistically significant, results from many plates have been averaged.

In the first group of experiments the X-rays used were the unfiltered general radiation from a Siemens Bucky tube having a tungsten target and a Lindemann glass window. The voltage across this tube was ca 12 KV peak and was provided by the unrectified output of a suitable transformer. A steady current of 8 MA was obtained by heating the

[1] Wyckoff, R. W. G., and Rivers, T. M., *J. Exp. Med.*, 1930, 51, 921.

cathode filament with an insulated storage battery. The bacteria were distant approximately 8 cm. from the tungsten target. Because of the long exposures required to give a sufficient killing action, only about 25 plates were irradiated in a day. Since the ratios of Table I and Text-fig. 1 are each averages of the counts upon 20 to 60 plates, several days were devoted to each experiment. These results obviously fall upon straight lines when plotted on semilogarithmic paper.

TABLE I

Survival Ratios with Bucky Tube

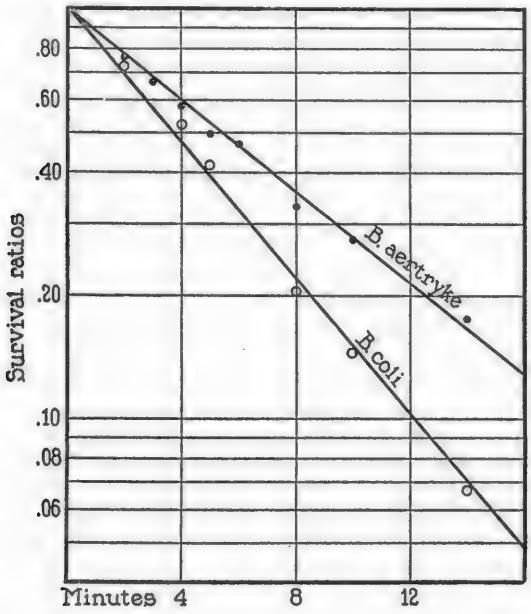
Time	Survival ratios	
	B. coli	*B. aertryke*
2 min.	0.750	0.768
3 "	—	.661
4 "	.526	.578
5 "	.416	.490
6 "	—	.466
8 "	.201	.329
10 "	.146	.274
12 "	.087	—
14 "	.064	.175

The survival ratios must therefore be expressed by an equation of the type

$$\frac{A_1}{A_0} = e^{-at}$$

Estimates of the intensity of the X-ray beam were made by measuring with a suitable chamber the ionization of air at the position of the irradiated bacteria. Subsequent experience has indicated that saturation currents were not obtained in these first experiments. No new determinations have been carried out because the general radiation of long wave length which constitutes the major output of a Bucky tube is poorly adapted to calculations of the mechanism by which cells are killed. It should be noted that the experiments of this group with *B. coli* and with *B. aertryke* were made under sufficiently different conditions so that no significance attaches to the relative killing rates found.

No commercially available electron-type X-ray tube supplies a monochromatic beam intense enough for experiments on the killing rates of bacteria. The self-rectifying gas tube developed in this laboratory and described[2] elsewhere is, however, suitable for this purpose and, equipped with a copper target, has been employed in a second series of experiments with *B. coli* and *B. aertryke.*



TEXT-FIG. 1. A plot of the survival ratios resulting from the use of soft general radiation from a tungsten target X-ray tube (Table I).

In some instances the radiation from this tube has been filtered only by the 0.001 inch aluminum forming its window; in others a thickness of 0.0009 inch metallic nickel foil was interposed between the bacteria and the target. This filter cuts down the general radiation and eliminates 99 per cent of the K-β line at the same time passing half of the K-α doublet. The tube was operated at about 4 MA and 34 KV peak (unrectified wave). During a single series of experiments the variations in the current through the tube were less than 0.1 MA and its output of X-rays was found to be steady to within 1 per cent.

[2] Wyckoff, R. W. G., and Lagsdin, J. B., *Radiology*, July, 1930, **15**, 42.

Each series required the exposure of about 100 plates, the results of which were averaged to give the survival ratios of Tables II and III. As Text-figs. 2 and 3 indicate, these ratios fall upon straight lines when plotted on semilogarithmic paper. Such a result agrees with the

TABLE II

Survival Ratios with Unfiltered Copper Radiation

Time	Survival ratios			
	B. coli (1)	*B. coli* (2)	*B. coli* (3)	*B. aertryke*
5 sec.	—	0.749	—	—
10 "	—	.617	0.645	0.793
20 "	0.358	.329	.548	.583
30 "	.233	.206	.422	.387
40 "	.167	.154	.344	.349
50 "	.112	—	.265	.270
60 "	.100	.089	.193	.234
Ionization current/sec./cm.^{3}.....	—	—	144.2 e.s.u.	—

TABLE III

Survival Ratios with Filtered Copper Radiation

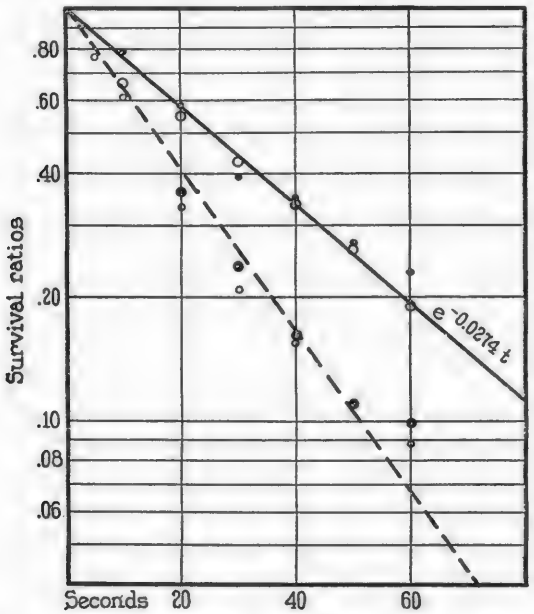
Time	Survival ratios		
	B. coli (1)	*B. coli* (2)	*B. aertryke*
20 sec.	0.667	0.711	0.804
40 "	.367	.574	.523
60 "	.345	.362	.391
90 "	.182	.291	.211
120 "	.109	.212	.189
Ionization current/sec./cm.^{3}.....	—	67.2 e.s.u.	67.2 e.s.u.

experiments of Holweck[3] and Lacassagne[3] on *B. pyocyaneus* with silver L-radiation.

The experimental arrangement is shown in Fig. 1. During use the stamping device for the standard area A and the irradiated area B replaced the diaphragm

[3] Holweck, F., *Compt. rend.*, 1929, **188**, 197; Lacassagne, A., *Compt. rend.*, 1929, **188**, 200.

C under the X-ray tube D. When set up, the tube D was carefully centered with the aid of a fluorescent screen to insure that the X-ray beam covering B should be uniform. A Petri dish carrying the desired number of bacteria spread upon its agar surface was placed on the stand E and raised by the remote control rod F until the knife edges of A and B cut through the agar. On opening the metal shutter G, the surface marked by B could then be given an exposure of desired length. After lowering E, it and the dish were removed with the help of F. This remote control was needed because the X-ray tube itself operated continuously

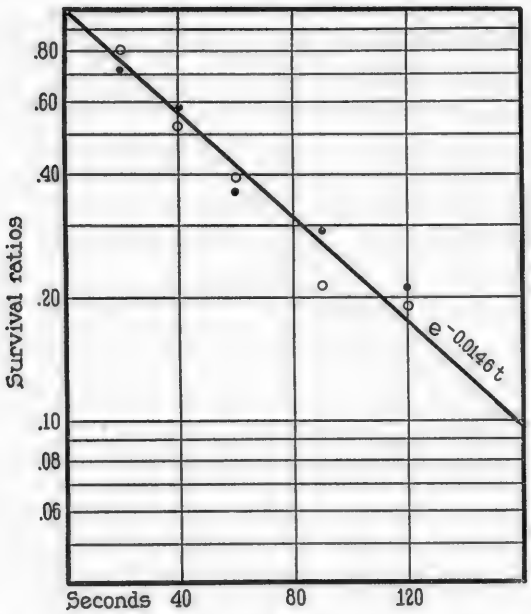


TEXT-FIG. 2. A plot of the data obtained by irradiating *B. coli* and *B. aertryke* with filtered copper rays. The small open, large black, large open and small black circles refer to experiments (1), (2), (3) and (4) of Table II.

throughout the entire experiment. When in use, the tube was covered by a lead housing such as that shown at H and during exposures the door K was kept closed. These precautions are essential to the safety of the experimenter.

The intensity of the X-rays striking the bacteria spread on the agar surface was obtained from measurements of the amount of ionization in air produced by these rays. Because of their absorbability, it was

considered inaccurate to employ for this purpose a small enclosed type ionization chamber. Accordingly, an open air chamber of standard design but of small size was made. Its over-all length was 7.0 cm. The actual length of the amber-insulated collecting electrode was 1.31 cm., its effective length 1.39 cm. The grounded electrodes on either side were each 2.54 cm. long. All three electrodes were 3.8 cm. wide. The charged shield surrounding and facing these plates was 3.2 cm.



TEXT-FIG. 3. A plot of data obtained by irradiating *B. coli* and *B. aertryke* with the same dose of unfiltered copper rays. The small black and large open circles refer to experiments (2) and (3) of Table III.

distant. On account of the extremely great ionizing power of the X-rays used, saturation in a chamber of this type can be obtained only if beams of small cross-section are measured. The currents produced in this chamber were measured by the usual balance methods[4] employing

[4] See for instance, Makower, W., and Geiger, H., Practical Measurements in Radio-activity, London, 1912, 15.

a variable voltage and a standard condenser of known capacity. This capacity was obtained by comparison against a small standard condenser and checked by calculation from its accurately determined dimensions.

The intensity of the X-rays striking a small area of the agar surface was found by replacing the stamping device AB of Fig. 1 with a small lead stop, C, having an opening 0.117 cm. in diameter. The air chamber was brought as near to the stop as possible (ca 3 mm.) and the ionization produced by the X-ray beam passing through it was ascertained. Knowing the length of the measured column of ionized air and its absorption coefficient under the conditions of temperature and pressure of the experiment and for copper rays, it is obviously possible to calculate the approximate ionizing power of the beam at the irradiated surface. A typical calculation will make clear the procedure followed and the approximations introduced.

Area of stop in position of bacterium = 0.0107 cm.^{2}
Height of air column the ionization of which is measured = 1.39 cm.
Corrected density of air = 0.001165
Temperature = 26°C., Barometer = 747 mm. Hg.
Absorption coefficient μ/ρ of air for copper radiation = 8.43
Balancing voltage on potentiometer = 18.8 V in 20 sec.
1 e.s.u. potential = 0.33×10^{-2} volts
Measured capacity of standard condenser = 321.4 cm.

Therefore the observed ionization current is

$$i' = \frac{18.8 \times 0.33 \times 10^{-2} \times 321.4}{20 \text{ sec.}} = 0.997 \text{ e.s.u.}$$

Since $I/I_0 = e^{-\mu l}$ where l is the length in centimeters of the absorbing column and $\mu = 8.43 \times 0.001165$, a column of air 1 cm. long will absorb 0.721 as much as a similar column of length 1.39 cm. Hence the ionization produced per second in 1 cm. depth of air by the rays striking 1 cm.^{2} of irradiated bacterial surface is taken as

$$i = \frac{i' \times 0.721}{0.0107} = 67.2 \text{ e.s.u. per cm.}^3 \text{ per sec.}$$

The intensities of the X-rays used in each of the three final standardized experiments when expressed in the foregoing units are recorded in Tables II and III.

Analysis

Existing knowledge of the properties of X-rays and of the mechanism of their absorption in inorganic matter makes it seem inevitable

that their absorption in bacteria is a quantized process. Thus the X-rays incident upon a cell will either pass through without altering it or else they will give up one or more quanta whose energy content is connected with the wave length λ of the rays through the familiar relation

$$E = h\nu = h\frac{\lambda}{c}$$

where h is Planck's constant, ν is the frequency of the rays and c is the velocity of light. It is known that a high velocity electron is liberated as a result of such an absorption. This electron gives rise to a chain of ions in the matter through which it passes and to X-rays which, in their turn, liberate more ions of less and less energy. The volume within which this cluster of ions resulting from a single quantum absorption is freed increases rapidly with the magnitude of the original quantum but, in matter having the density of a bacterium, it is at greatest only a very small fraction of a cubic millimeter. The changes X-rays produce in protoplasm are naturally identified with the physico-chemical changes induced by this ionic shower.

The physical consequences of the absorption of an X-ray quantum so closely resemble those attending the direct absorption of a high velocity electron that it seems permissible to treat the killing action of X-rays by the same statistical analysis which has previously been used in studies of the killing of bacteria by cathode rays.

On this basis, the straight line obtained by plotting survival ratios upon semilogarithmic paper means that, on the average, the death of a bacterium is the result of the absorption of a single X-ray quantum. As the earlier analysis[5] has shown, survival ratios under these conditions can be calculated from the expression

$$\frac{A_1}{A_0} = e^{-\alpha t}$$

where t is the time and α is the expected, or average, number of quantum absorptions in unit time.

A more intimate picture of the killing process is to be had from a study of this expected number of hits. Under conditions of satura-

[5] Wyckoff, R. W. G., and Rivers, T. M., *op. cit.*

tion, the air ionization chamber measures the total number of ions produced by the quanta absorbed in a known volume of air. If the number of pairs of ions liberated by a single quantum can be judged, then the number of quanta absorbed in this volume of air is known. Furthermore, if the ratio of the absorption coefficients of air and of a bacterium can be estimated, the *average* number of quanta absorbed by a single bacterium in unit time is readily computed from the size and shape of the organism. This has been done for experiments with filtered and unfiltered copper rays.

The following typical calculations based upon the standardized experiment of Table III using *B. aertryke* will show how the average number of hits has been obtained and will make clear the approximations that have been introduced in the process.

$$67.2 \text{ e.s.u.} \approx 67.2 \times 0.21 \times 10^{10} \text{ ion pairs/sec.} = 1.411 \times 10^{11} \text{ ion pairs/sec./cm.}^3$$

A small amount of radiation is absorbed in the air between the diaphragm C and the collecting electrode of the air chamber. This absorption is readily calculated from the dimensions of the apparatus and the absorption coefficient ($\mu/\rho = 8.43$) of copper radiation. With the resulting correction it is found that 1.46×10^{11} ion pairs will be produced per second per cm.^{3} at the position of the irradiated bacteria. In the absence of direct experimental knowledge of the absorption coefficient of protoplasm, the mass coefficients of air and of living cells are customarily assumed equal. If this assumption, which cannot depart far from the truth, is made, the amount of absorption in 1 cm. thickness of air is about 27.9 times that of one bacterium. Both the *B. coli* and the *B. aertryke* have been taken as rods 0.5μ in diameter and 2μ long. Computation shows that to a first approximation one bacillus may be considered equivalent in absorption to a rectangular block of protoplasm 1×10^{-8} cm. in area, 0.42μ thick and of unit density. From these quantities the average number of ion pairs absorbed per bacterium per second becomes

$$\frac{1.46 \times 10^{11} \times 1 \times 10^{-8}}{27.9} = 52.3 \text{ ion pairs/sec./bacterium.}$$

In order to obtain the number of X-ray quanta absorbed per second it is necessary to know how many ions are liberated when one quantum is absorbed in air. The best available measurements[6] indicate that for X-rays of the quality used in these experiments about 35 volts are required to produce each electron pair. The voltage equivalent of the K-α lines of copper as computed from the familiar quantum relation

[6] Kulenkampff, H., *Ann. d. Physik*, 1926, **79**, 97.

Voltage (in KV) = $12.34/\lambda$(in A) = $12.34/1.537$

is 8.029 KV. From this the number of ion pairs arising through the absorption of one quantum of Cu K-α radiation is

$$8029/35 = 229.$$

The average number, α, of quantum absorptions per second then is

$$52.3/229 = 0.228.$$

The observed survival ratios in Table III lead to a straight line having the equation $A_1/A_0 = e^{-0.0146t}$ (Text-fig. 2). It will be noted that this experimental $\alpha' = 0.0146$ is much smaller than the α calculated from ionization measurements.

DISCUSSION

Accepting the foregoing type of analysis as an essentially correct, if rough, description of what happens when X-rays strike bacteria, the smallness of the ratio α'/α when taken in connection with the linearly exponential character of the experimental results shows that though on the average the absorption of one quantum of these radiations is sufficient to kill a bacterium of either *B. coli* or *B. aertryke*, relatively few of the absorbed quanta are lethal. In the experiment just calculated 0.0146/0.228 or about one in 15.6 kills. In the standardized experiment of Table II using *B. coli* one in 17.8 is deadly ($\alpha = 0.489$, $\alpha' = 0.0274$). This agreement is satisfactory since it is scarcely to be expected that the results with filtered and unfiltered rays should be identical.

The fact that so many quanta can be absorbed by a bacterium without causing death apparently means that the vital elements within the cell which can be destroyed by a direct quantum hit are much smaller than the cell itself. If the volume through which the quantum acts were negligible compared to that of the vital element of the cell and if there were only one such element in a bacterium, then the "sensitive volume" that would have to be hit in order to bring about death would be α'/α. In the two standardized experiments with filtered rays this ratio is 0.064; in the series using unfiltered radiation it is 0.056. The spheres of action of these quanta cannot, however, be disregarded and the quantities α'/α are to an important degree measures of these regions of quantum action. Furthermore, if, as seems natural, the

vital parts of the cell are identified with its chromatin material, more than one "sensitive volume" exists within it. It can nevertheless be concluded that the volume of the "vital elements" contained within one of the bacteria studied scarcely exceeds 0.05 of its entire volume. This estimate seems to be in serious conflict with the earlier conclusions of Holweck and Lacassagne with *B. pyocyaneus*. The published data are, however, too few to allow of a satisfactory comparison.

Within the limits of experimental error there is no difference between the sensitiveness of *B. aertryke* and *B. coli* to copper K-radiation (Text-fig. 2). A similar result was found when studying the killing action of cathode rays upon them.

Additional experiments are being carried out to ascertain in what way differences in X-ray wave length affect the killing of these organisms.

Valuable help in the carrying out of these experiments has been given by Charles G. Porskieves.

CONCLUSIONS

Both copper K X-rays and the soft general radiation from a tungsten tube operated at 12 KV kill *B. coli* and *B. aertryke* in a linearly exponential fashion. Within the experimental limits, the two organisms appear to be equally sensitive to these radiations.

By making use of the fact that X-ray energy is absorbed in quanta, an approximate picture can be formed of the mechanism of this destructive action. If the average numbers of quanta (α) absorbed per bacterium per second are calculated from measurements of air ionization using the quantities outlined in the text, survival ratios for these bacilli can be approximately represented by the equations

$$\frac{A_1}{A_0} = e^{-0.064\,\alpha\,t}$$

for filtered copper rays and

$$\frac{A_1}{A_0} = e^{-0.056\,\alpha\,t}$$

for unfiltered copper rays (peak voltage = 34 KV).

In terms of the foregoing interpretation this means that when death results, it is caused by the absorption of a single X-ray quantum of energy. Since only about one in twenty of the absorbed quanta kills, the sensitive cell constituents whose destruction leads to cell death must have a volume which is less than 0.06 of the bacterium itself.

EXPLANATION OF PLATE 17

FIG. 1. A photograph of the experimental arrangement for irradiating bacteria.

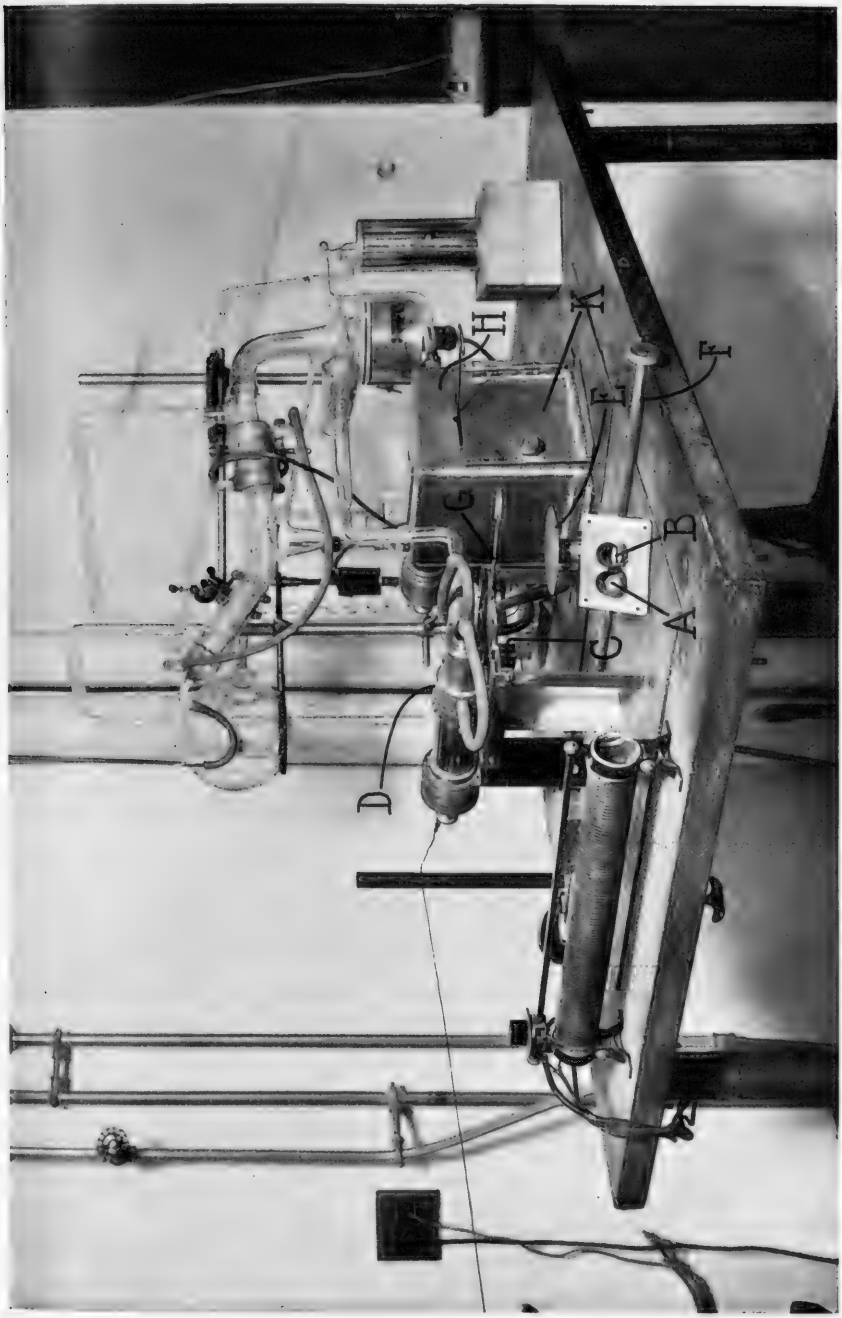


FIG. 1

(Wyckoff: Killing of certain bacteria by X-rays)

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THE CATALYTIC EFFECT OF METHYLENE BLUE ON THE OXYGEN CONSUMPTION OF TUMORS AND NORMAL TISSUES

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It has previously been shown (1, 2, 3) that the addition of methylene blue, or similar reversibly oxidized and reduced dyes, increases the oxygen consumption of cell suspensions. It was also demonstrated that correlated with this increase, there is a decrease in the aerobic glycolysis* as shown by a diminished lactic acid formation (4). It was furthermore suggested (5) that the effect of the dye seemed to be proportional to the fermentative power of the cell. To test the validity of this hypothesis, use has been made of the fundamental researches of Warburg (6) on the metabolism of normal tissues and tumors. Warburg has shown there is a sharp difference between the metabolism of tumors and that of normal adult tissues. While in general normal adult tissues possess a high respiration, sufficient to check the appearance of the fermentative processes, tumors, on the other hand, have a low respiration, and consequently part of the energy necessary for the performance of cellular activities has to be provided by fermentation. The lactic acid produced in tumors may thus reach about 13 per cent of the weight of the tumor. If methylene blue and similar dyes act as catalysts for the oxidative processes of living cells only when the cells in question possess a fermentative power, these two kinds of tissues are the most appropriate material with which to test the validity of the hypothesis.

The experiments were performed on fresh rat tissues and rat tumors taken soon after death caused by a blow on the head. The tissues and tumors were cut with

* Whenever the word glycolysis is employed in this paper, it is used in the sense given by Warburg, *i.e.*, the splitting of one molecule of glucose into two molecules of lactic acid according to the equation $C_6H_{12}O_6 = 2C_3H_6O_3$.

a hand razor into thin slices, 0.4 mm. thick, to allow free diffusion of oxygen throughout the section. They were kept in a saline solution containing 0.15 gm. per cent of glucose, and a mixture of phosphate buffers, $\frac{M}{15}$ having a pH of 7.38. The oxygen consumption was determined in Warburg vessels with Barcroft manometers. The temperature of the water bath was 37.5°C.

The Effect of Methylene Blue on the Oxygen Consumption of Normal Tissues

The following normal tissues were employed: liver, kidney, brain (gray matter), spleen, and testicles. For the study of pancreas, rabbits were used. The respiration and carbohydrate metabolism of slices of these tissues have been determined *in vitro* by Warburg and his associates. Liver, pancreas and kidney possess no aerobic glycolysis. Brain and testicles are reported as having a small aerobic glycolysis. The metabolism of spleen has been determined by Murphy and Hawkins (7) and found to show an aerobic glycolysis.

In preliminary determinations, the metabolism of all these tissues was measured by the use of the well known Warburg methods, and found to be more or less like that reported by the above named investigators, except for the metabolism of rat testicles, where a $Q\overset{O_2}{O_2}$ of -15.0 was found, and a $Q\overset{O_2}{CO_2}$ of $+1.2$ instead of $Q\overset{O_2}{O_2} - 12.3$ and $Q\overset{O_2}{CO_2} + 7.2$ found by Warburg. In view of this discrepancy, the glucose consumption and lactic acid formation of rat testicles were determined chemically. As it can be seen from Table 1 there was no lactic acid formation, though there was destruction of glucose. The addition of methylene blue did not have any influence either on the speed of glucose consumption or in the lactic acid formation.

TABLE 1

The Effect of Methylene Blue on the Glucose Consumption and Lactic Acid Formation of Rat Testicles

Testicles continuously shaken in a saline solution containing phosphate buffer $\frac{M}{15}$ pH 7.38.

437.8 mg. testicle	Glucose in mg. per cent	Lactic acid in mg. per cent
Before incubation	114.0	66.9
2 hours after incubation control	87.0	66.4
M. B. added	87.0	66.8

TABLE 2

The Effect of Methylene Blue on the Oxygen Consumption of Normal Tissues

Kind of tissue	O_2 consumption in c.mm. per hour		Per cent increase or decrease
	Before dye addition	After dye addition	
Rat kidney:			
1)	47.6	47.3	−0
2)	128.8	116.3	−9.7
3)	59.0	56.2	−4.7
4)	51.6	38.0	−26.3
5)	63.0	46.5	−26.2
6)	94.0	79.0	−15.9
Rat liver:			
1)	37.2	37.5	−0
2)	80.6	75.0	−6.9
3)	41.7	40.3	−3.3
4)	42.7	37.4	−12.4
5)	54.2	52.2	−3.7
Rabbit pancreas:			
1)	25.6	23.4	−8.6
2)	22.0	20.6	−6.8
3)	17.5	13.0	−25.7
4)	23.6	19.0	−19.5
5)	24.8	22.6	−8.9
Rat testicle:			
1)	120.5	119.5	−0
2)	118.9	114.9	−3.4
3)	117.2	114.0	−2.7
4)	140.7	140.0	−0
Rat spleen:			
1)	31.0	33.4	+7.7
2)	29.7	30.8	+3.7
3)	38.3	42.1	+9.9
4)	32.0	34.2	+6.9
5)	34.8	37.0	+6.3
6)	55.1	55.4	0
7)	46.8	57.4	+22.7
Rat brain (gray matter):			
1)	28.6	30.3	+6.0
2)	35.3	38.6	+9.3
3)	37.2	37.6	0
4)	33.0	41.8	+26.6
5)	25.8	31.9	+23.6
6)	28.8	33.2	+15.3
7)	29.2	31.7	+8.5

In Table 2 have been tabulated the results of the experiments on the oxygen consumption of normal tissues, and the effect of methylene blue. The values given do not refer to the weight of the tissue, but are the figures directly found. The oxygen consumption of these tissues, weighed after drying for some hours at 100°C., was found to correspond to the figures given by Warburg. In the tissues where there is no aerobic glycolysis, *i.e.*, lactic acid production, methylene blue has no catalytic effect; moreover, it exerts a definite toxic action as shown by a definite drop in the oxygen consumption. Methylene blue increases the oxygen consumption of brain tissue and spleen, which possess an aerobic glycolysis of $Q\frac{O_2}{CO_2}$ +2.5 and +2.3 respectively. Methylene blue does not increase the oxygen consumption of the tissue of rat testicles, nor does it have action on the lactic acid formation. It is interesting to note that the toxic effect of the dye observed in kidney and liver tissues is not found in testicles.

In order to provide further evidence of the action of methylene blue on the fermentation phase of cellular metabolism, the oxygen consumption of the above mentioned tissues was measured in the presence of KCN ($\frac{M}{300}$), previously neutralized to the same pH as that of the saline solution, and the effect of methylene blue determined afterwards. Cyanide is a specific respiratory poison as shown by Warburg, but it has no effect on the fermentative process which proceeds undisturbed. Incidentally, Dixon and Elliot's (8) observations on the influence of cyanides to inhibit partially the power of normal tissues to consume oxygen were confirmed.* While KCN inhibits completely the respiration of goose erythrocytes, yeast cells and *Arbacia* and starfish eggs, this inhibition is never complete in the case of normal tissues. In Table 3 are given average figures of the experiments. Liver, kidney, pancreas, and testicles, which normally did not show any increase in their oxygen consumption by the action of methylene blue, exhibit a manifest increase when the respiration is inhibited by cyanide and the glycolysis appears. In spleen, the increase of the oxygen

* After this paper had been sent to press, Howard, from Warburg's laboratory, showed that KCN inhibition of respiration is about complete when the tissues are kept in Ringer solution buffered with bicarbonate (*Biochem. Z.*, 1930, **221**, 498). This recent communication does not affect the experiments related here.

consumption by the action of methylene blue is considerably enhanced by previous addition of cyanide.

Wendel (9) has reported that red blood corpuscles oxidize added sodium lactate in the presence of methylene blue. The addition of lactates to normal tissues, where the catalytic power of the dye is absent, produces neither an increase on the oxygen consumption nor a diminution of the added lactates. The determinations were made with liver, kidney and testicles, both by the manometric methods and by chemical analysis.

TABLE 3

The Effect of Methylene Blue on the Oxygen Consumption of Normal Tissues Previously Treated with KCN ($\frac{M}{300}$)

Kind of tissue	O_2 consumption per hour in c.mm.		Per cent increase
	Before dye addition	After dye addition	
Rat kidney	22.9	36.3	58.5
Rat liver	13.8	16.4	18.8
Rat spleen	5.2	20.1	286.4
Rat testicle	9.2	27.6	200.0

The Effect of Methylene Blue on the Oxygen Consumption of Tumors

As a result of the observations of Warburg, repeated and confirmed by a number of investigators, it is known that tumor tissues possess a metabolism different from the metabolism of normal adult tissues. Tumors, whether taken from animals or cultivated *in vitro* show in general a low respiration compensated by an abnormally high fermentation, which is more manifest when measuring the "Pasteur Reaction" of these tissues, *i.e.*, the excess fermentation given by the following formula $= U = Q\,{}^{N_2}_{M} - 2\,(QO_2)$ where $Q\,{}^{N_2}_{M}$ is the anaerobic glycolysis and QO_2 the respiration. Let us add that aerobic glycolysis is not a specific feature of tumor tissues. Warburg (10) has summarized examples of normal tissues possessing aerobic glycolysis, Crabtree (11) has shown it to be a property of certain pathological overgrowths associated with intracellular viruses, and recently Neuhaus (12) has reported the presence of an appreciable aerobic glycolysis in granulation tissues.

TABLE 4

The Effect of Methylene Blue on the Oxygen Consumption of Tumors

Kind of tumor	O_2 consumption in c.mm. per hour		Per cent increase
	Before dye addition	After dye addition	
Human carcinoma (breast):			
1)	17.0	21.3	25.3
2)	24.6	33.7	37.0
3)	26.0	31.0	19.2
Rat carcinoma (Walker 256):			
1)	24.4	40.5	66.0
2)	22.5	29.5	31.1
3)	32.4	40.3	24.4
4)	29.2	42.3	44.8
5)	16.3	33.6	106.0
6)	24.3	38.4	58.0
7)	25.1	36.5	45.4
8)	32.0	48.0	50.0
9)	12.7	21.9	72.4
10)	28.1	46.3	64.8
11)	32.3	48.5	50.2
Rat adenocarcinoma No. 20:			
1)	14.3	25.6	79.0
2)	25.9	45.4	75.2
3)	19.7	37.3	89.3
Rat sarcoma No. 1 (Walker):			
1)	24.1	33.3	38.2
2)	26.6	40.3	51.5
3)	32.9	48.8	48.3
4)	35.3	47.4	34.3
5)	49.7	62.8	26.4
Rat sarcoma No. 135:			
1)	38.7	57.0	47.3
2)	30.5	42.5	39.3
3)	41.3	54.5	32.0
Rat sarcoma No. 10:			
1)	29.9	41.0	37.1
2)	34.4	53.9	56.7
Chicken sarcoma (Rous):			
1)	11.6	23.5	102.0
2)	10.5	22.7	116.0
3)	9.4	19.6	108.6
4)	17.4	27.0	55.2
5)	10.1	14.2	39.4

It was expected that methylene blue would exert its catalytic power in the presence of tumors, since they possess a high aerobic glycolysis. Table 4 demonstrates that such is the case. Different kinds of tumors were used in these experiments: human carcinoma, rat sarcoma, rat adenocarcinoma, and Rous chicken sarcoma with the same results, namely that there is a definite increase in the oxygen consumption of these tissues in the presence of methylene blue, an increase which is higher than the one observed in brain and spleen.

When the respiration is inhibited by cyanides, the addition of methylene blue, although increasing considerably the oxygen consumption in relation to the previous low value obtained before the

TABLE 5

The Effect of Methylene Blue on the Oxygen Consumption of Tumors, Previously Treated with KCN ($\frac{M}{300}$)

Kind of tumor	O_2 consumption per hour in c.mm.		Per cent increase
	Before dye addition	After dye addition	
Human carcinoma	1.5	7.1	373.4
Rat adenocarcinoma (Walker No. 76)	0	15.0	∞
Rat carcinoma, mammary gland (Walker No. 256)	0.5	7.7	1440.0
Rat sarcoma (Walker No. 1)	3.8	16.5	337.0

dye is added, does not bring it back to the values observed without cyanides. There is in the action of cyanides on the oxygen consumption of tumors an interesting phenomenon which will be studied more carefully in this laboratory. KCN inhibits the oxygen consumption of tumors almost completely. In the experiments here reported the inhibition was from 100 per cent to 90 per cent in relation to the oxygen consumption of the same weight of untreated tumor. This observation is in sharp contrast to the findings of Dixon and Elliot (8), which have been confirmed in this laboratory, of the incomplete inhibition of the oxygen consumption of normal tissues by cyanides, which, in the experiments reported in Table 3, was from 70 to 85 per cent.

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2)	22.5	29.5	31.1
3)	32.4	40.3	24.4
4)	29.2	42.3	44.8
5)	16.3	33.6	106.0
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8)	32.0	48.0	50.0
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10)	28.1	46.3	64.8
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1)	14.3	25.6	79.0
2)	25.9	45.4	75.2
3)	19.7	37.3	89.3
Rat sarcoma No. 1 (Walker):			
1)	24.1	33.3	38.2
2)	26.6	40.3	51.5
3)	32.9	48.8	48.3
4)	35.3	47.4	34.3
5)	49.7	62.8	26.4
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2)	30.5	42.5	39.3
3)	41.3	54.5	32.0
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2)	10.5	22.7	116.0
3)	9.4	19.6	108.6
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The Relation between the Catalytic Power of Methylene Blue and the Aerobic Glycolysis of Cells and Tissues

In an attempt to correlate, in a quantitative way, the relation between the catalytic power of methylene blue, and the fermentative power of the tumors studied, the aerobic glycolysis was determined, employing the methods of Warburg. The data have been tabulated in Table 6. The figures given for the aerobic glycolysis of normal tissues have been taken from Warburg's data (13), excepting that the aerobic glycolysis of spleen has been taken from the data of Murphy and

TABLE 6

Relation between the Aerobic Glycolysis, and the Catalytic Power of Methylene Blue on the O_2 Consumption of Tumors and Normal Tissues

Kind of tumor or tissue	Per cent increase or decrease due to M. B. (O_2 consumption)	Aerobic glycolysis
Kidney	−13.8	0
Liver	−5.2	0
Pancreas	−13.9	0
Spleen	+8.2	+2.3
Brain	+12.7	+2.5
Human carcinoma	+27.2	+9.2
Rat carcinoma (Walker)	+55.7	+17.7
Rat adenocarcinoma (Walker)	+81.7	+22.5
Rat sarcoma (Walker No. 1)	+39.7	+11.3
Rat sarcoma (Walker No. 135)	+39.5	+12.1
Chicken sarcoma (Rous)	+84.2	+21.4

Hawkins. The data on the aerobic glycolysis of Rous chicken sarcoma have also been taken from the last mentioned authors. It can be seen in this table that methylene blue exerts its catalytic power only on cells or tissues possessing aerobic glycolysis. There is evidently a rough proportionality between the catalytic power of the dye and the fermentative power of the cell or tissue.

DISCUSSION

It has been established (5) that the following factors influence the catalytic power of reversibly oxidized and reduced dyes on the cellular oxidative processes: the permeability of the cell membrane to the

dye; and the reduction potential of the dye, since the speed of this activation is a function of the speed at which the dye is reduced by the cell, and the speed at which the leucodye is oxidized by the atmospheric oxygen. From the experiments reported in this paper it can be said that the fundamental factor is the fermentative power of the cell or tissue. Thus normal adult tissues have, in so far as the microscopic sections can reveal, the same permeability for methylene blue as tumors; they possess the same reducing power as shown by Voegtlin, Johnson and Dyer (14). Nevertheless, while the dye has no action or even exerts a toxic effect on the respiration of normal adult tissues when deprived of aerobic glycolysis, the dyes show their catalytic power on cells or tissues possessing a fermentative power as expressed by their aerobic glycolysis. Methylene blue has no effect on the oxygen consumption of most normal adult tissues because they do not produce lactic acid under aerobic conditions. The high respiration of the cell checks the fermentative power. When the respiration of these tissues is inhibited, by the addition of a specific respiratory poison, namely KCN, and the fermentation appears, methylene blue exhibits its catalytic power, increasing the oxygen consumption of these tissues, normally inactive to the action of the dye.

The effect of methylene blue on the oxygen consumption of tumors is a further confirmation of this point of view. It is known that the respiratory power of the tumors is limited; as a consequence, and, according to the brilliant conceptions of Pasteur (15), because respiration is performed at the expense of fermentation, the fermentative process in aerobic conditions appears to provide the energy necessary for the maintenance of the cellular metabolism. Neoplasms therefore provide the necessary conditions for the action of methylene blue. An extremely interesting question is raised here, a problem which will be investigated shortly. If it is possible to change the metabolism of tumor tissues by the addition of methylene blue in such a way that the tissue shows an increased respiration, as well as a decreased aerobic glycolysis with a consequent shift of the Pasteur Reaction towards the negative side, it means, that, by the action of the dye, the metabolism of these tumors is shifted towards the direction of the metabolism of normal adult tissues. By using a small concentration of methylene blue on cultures of tumors, and growing them for several generations in a medium containing the dye, possibly the characteristic

tumor metabolism, with its low respiration and high fermentation, might be changed permanently into one showing the characteristics of normal tissues.

The constant relation between the fermentative power of cells and tissues, and the increase of their oxygen consumption after the addition of methylene blue is so general and has been tested with such a variety of cells and tissues, that it seems justifiable to suggest the use of methylene blue as a test for the fermentative power of cells and tissues.

CONCLUSIONS

1. Methylene blue has no catalytic effect on the oxygen consumption of those normal adult tissues which do not possess aerobic glycolysis. The dye increases the oxygen consumption of these tissues when their respiration has been inhibited by the addition of KCN and their fermentative power thus brought into action.

2. Methylene blue increases the oxygen consumption of normal tissues having aerobic glycolysis, and of tumors.

3. The effect of methylene blue is roughly proportional to the fermentative power of tissues.

I wish to express my sincere thanks to Dr. Margaret Reed Lewis of the Carnegie Laboratory of Embryology for generously supplying me with the rat tumors used in these experiments.

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